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Awarding institution:
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female sterile (1) homeotic controls metabolic and immune function and enhances survival via AKT and FOXO

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This thesis is submitted to King's College London
for the degree of Doctor of Philosophy

September 2017

Abstract

The *Drosophila melanogaster* fat body, analogous to mammalian adipose tissue and the liver, is the primary organ of fat and glycogen storage as well as being responsible for the humoral immune response following infection. Normal functioning of the fat body is of critical importance to the survival of the organism, but many molecular regulators of its function remain ill-defined. Here, for the first time, we demonstrate that the *Drosophila* bromodomain-containing protein *fs(1)h* is essential in the fat body for normal lifespan as well as metabolic and immune homeostasis. Under physiological conditions, flies lacking *fs(1)h* in the fat body exhibit a severely reduced lifespan, abnormally high expression of immune target genes including antimicrobial peptides (AMPs) and cytokines, an inability to utilise triglycerides following periods of starvation, and low basal AKT activity, mostly resulting from systemic defects in insulin signalling. Loss of *fs(1)h* in the fat body also results in hypoglycemia and a dysregulation of several fat body-derived signals, indicating a role for fat body *fs(1)h* in the regulation of various systemic endocrine signals. Removing a single copy of the AKT-responsive transcription factor *foxo* ameliorates almost all the observed phenotypes, restoring lifespan, metabolic function, uninduced immune gene expression, and AKT activity suggesting many of the *in vivo* effects of *fs(1)h* in the fat body are *foxo*-dependent. However, survival is not rescued and AMP expression is still elevated following bacterial infection in *fs(1)h* knockdown *foxo* heterozygous flies, indicating some of the phenotypes observed are independent of the FOXO hyperactivation. We propose that the promotion of systemic insulin signalling activity is a key *in vivo* function of fat body *fs(1)h*.

Acknowledgements

I am grateful to Marc Dionne and my PhD committee for their advice and supervision over the past 4 years.

I would like to thank Katie and Katrin for their invaluable support, both scientifically and non-scientifically; I wouldn't have been able to do it without either of you. Thanks also to the members of CIBCI at King's College and the members of the South Kensington Fly Room at Imperial College.

To my parents, thank you for your unconditional support and always believing in me, I will be forever grateful.

This PhD was funded by BBSRC and GlaxoSmithKline.

Declaration

I confirm that the work included in this thesis is my own. Any collaborative work or information from other sources included in this thesis has been referenced accordingly.

Jessica Sharrock
September 2017

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Abbreviations

2-NBDG	2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose
4E-BP	Eukaryotic translation initiation factor 4E binding protein 1 (*Drosophila* name: Thor)
5caC	5-Carboxylcytosine
5fC	5-Formylcytosine
5hmC	5-Hydroxymethylcytosine
5mC	5-Methylcytosine
20E	20-hydroxyedysone, ecdysone
Acc	Acetyl-CoA carboxylase
ACF	ATP-dependent chromatin assembly factor large subunit
Actβ	Activin-β
AGM	Aorta-gonad-mesonephros
AKH	Adipokinetic hormone
AKH-R	Adipokinetic hormone receptor
Akt	Also known as Protein Kinase B (PKB)
AMPs	Antimicrobial peptides
ANK	ANKyrin repeats
AP-1	Activator protein 1
ARID	AT-rich interaction domain
ATF-2	Activating transcription factor 2
ATGL	Adipocyte triglyceride lipase
ATP	Adenosine triphosphate
Atta	Attacin
Babo	Baboon
BBR	Brilliant Blue R
BCP	Bromodomain-containing protein
BD	Bromodomain
BET	Bromodomain and extraterminal domain
BHI	Brain heart infusion media
bmm	Brummer
BMP	Bone morphogenetic protein
bon	bonus
Br140	Bromodomain-containing protein 140
BRD2/3/4	Bromodomain-containing protein 2/3/4

Brd8	Bromodomain-containing protein 8
Brg1	Brahma-related gene 1
brm	brahma
BSA	Bovine serum albumin
Bsk	Basket
CAM	Cerium Ammonium Molybdate
CBP	CREB-binding protein
CCHa2	CCHamide-2
CCHA2-R	CCHamide-2 Receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
CecA1	Cecropin A
ChIP	Chromatin immunoprecipitation
CNS	Central nervous system
CO_2	Carbon dioxide
Co-IP	Co-immunoprecipitation
CREB	cAMP response element binding
CTM	C-terminal motif
crq	croquemort
CYLD	Cylindromatosis
DAP	diaminopimelic acid
DAPI	4’,6-diamidino-2-phenylindole
daw	Dawdle
Def	Defensin
dH_2O	Distilled water
Dif	Dorsal-related immune factor
DILP	*Drosophila* insulin-like peptide
Dipt	*Diptericin*
DNA	Deoxyribonucleic acid
dnr1	defense repressor 1
dNTP	Deoxynucleotide
DMSO	Demethyl sulfoxide
dome	domeless
dpp	decapentaplegic
Dpt	Diptericin
Dredd	Death related ced-3/Nedd2-like caspase

Dro	Drosocin
Drs	Drosomycin
DTT	Dithiothreitol
E2F	E2 factor
E(bx)	Enhancer of bithorax
EcR	Ecdysone receptor
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
eGFP	Enhanced green fluorescent protein
EMT	Epithelial-mesenchymal transition
ET	Extraterminal domain
Fadd	Fas-associated death domain
Fas	Fatty acid synthase
FBS	Fetal bovine serum
FBXL	F-box and leucine rich repeat
FGF	Fibroblast growth factor
FOXO	Forkhead box transcription factor
FOXP3	Forkhead box P3
F. novicida	*Francisella novicida*
fs(1)h	female sterile (1) homeotic
g	grams
Gal80^{ts}	Temperature sensitive Gal80
Gbb	Glass bottom boat
Gbp1/Gbp2	Growth-blocking peptide 1/2
GCM/GCM2	Glial cells missing/ Glial cells missing 2
Gcn5	Histone acetyltransferase Gcn5
GFP	Green fluorescent protein
Gp130	Glycoprotein 130
GPCR	G-protein coupled receptor
H_2O	Water
H_2SO_4	Sulphuric acid
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HIV	Human immunodeficiency virus
hkb	hückebein
Hml	Hemolectin

hop	hopscotch
Hox	Homeobox
hpRNA	Hairpin ribonucleic acid
HPV	Human papilloma virus
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cell
Hsl	Hormone sensitive lipase
Hz	Hertz
IBET	BET inhibitor
IFN-γ	Interferon-gamma
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IIS	Insulin/insulin-like growth factor signalling
IκB/IKK	Inhibitor of kappa B
IKK-γ	I kappa B Kinase-gamma
IL	Interleukin
IL-1β	Interleukin-1 beta
IL-1R	Interleukin-1 receptor
Imd	Immune deficiency
ImpL2	Ecdysone-inducible gene L2
InR	Insulin-like receptor
IPC	Insulin producing cell
IR	Inverted repeats
IRD5	immune-response deficient 5
Jak	Janus kinase
JARID	Jumonji, AT-rich Interactive Domain
JNK	Jun N-terminal kinase
Jmj	Jumonji
JMJD	Jumonji domain
JDCP	Jumonji domain-containing protein
K	Lysine
KAc	Potassium actinium
κB	kappa B
kDa	kilodalton
Kdm	Lysine (K)-specific demethylase
kn	knot

LD	Lipid droplet
LDS	Lithium dodecyl sulfate
LiCl	Lithium chloride
lid	Little imaginal discs
L. monocytogenes	*Listeria monocytogenes*
LPS	Lipopolysaccharide
Lz	Lozenge
M	mole
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MAPKKK	Mitogen-activating protein kinase kinase kinase
MCAP	Mitotic chromosome-associated protein
M-CSF	Macrophage colony-stimulating factor
MD-2	Myeloid differentiation protein-2
mAMP	milliampere
Me	Methylation
Mef2	Myocyte enhancer factor 2
MES	2-ethanesulfonic acid
$MgCl_2$	Magnesium chloride
ml	millilitre
mM	millimole
mRNA	Messenger RNA
Mtk	Metchnikowin
MyD88	Myeloid differentiation primary response gene 88
Myo	Myoglianin
NA	Numerical Aperture
n.d	Non-detected
nej	nejire
NF-κB	Nuclear factor κ light chain enhancer of activated B cells
nl	nanolitre
NLC	No lard control
NLR	Nod-like receptor
nm	nanometre
OD	Optical density
pAKT	Phosphorylated AKT
PB	polybromo

PBS	Phosphate buffered saline
PCAF	P300/CBP-associated factor
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent kinase 1
Pepck	Phosphoenolpyruvate carboxykinase
PFA	Paraformaldehyde
PGN	Peptidoglycan
PGRP	Peptidoglycan recognition protein
PGRP-LC	Peptidoglycan recognition protein LC
PHF	Plant homeodomain finger
PI3K	Phosphoinositide-3-kinase
pirk	Poor Imd response upon knock-in
plin1	perilipin 1 (also known as lipid storage droplet 1, Lsd-1)
plin2	perilipin 2 (also known as lipid storage droplet 2, Lsd-2)
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptor
PSR	Phosphatidylserine receptor
PTEFb	Positive transcription elongation factor b
PVDF	Polyvinylidene difluoride
PVF	PDGF/VEGF factor
PVR	PDGF and VEGF receptor
RCC	Renal cell carcinoma
RCF	Relative centrifugal force
Rel	Relish
Rheb	Ras homolog enriched in brain
RIPA	Radioimmunoprecipitation assay buffer
RT	Room temperature
RT-qPCR	Real time quantitative polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
RNA	Ribonucleic acid
RNA-seq	RNA-sequencing
RNAi	Ribonucleic acid interference
ROS	Reactive oxygen species
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute Medium
S6K	S6 Kinase

Sax	Saxophone
Scw	Screw
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SH2	Src Homology 2
siRNA	Small interfering ribonucleic acid
SIRT6	Sirtuin-6
SPE	*Spätzle*-processing enzyme
spz	spätzle
Socs36E	Suppressor of cytokine signalling 36E
Srp	Serpent
STAT	Signal transducer and activator of transcription
SWI/SNF	SWItch/Sucrose Non-Fermentable
TAF-1	TBP-associated factor-1
TAG	Triacylglycerol (also known as triglyceride)
TAK1	Transforming growth factor β-activated kinase 1
TBP	TATA-binding protein
tbrdp	Testis-specifically expressed BCP
TBS-T	Tris-buffered saline + Tween20
TE	Tris-EDTA buffer
TEP-1	Thiolester-containing protein-1
Tg	Transgenic
TGF-β	Transforming growth factor-β
Tkv	Thickvein
TLC	Thin layer chromatography
tll	tailless
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNF-α	Tumour necrosis factor alpha
TNF-R	Tumour necrosis factor Receptor
TOR	Target of rapamycin
tou	toutatis
TotA	Turandot peptide A
Trbd	trabid
trx	trithorax
TSB	Tryptic soy broth

TSC1/2	Tuberous sclerosis proteins 1/2
UAS	Upstream activation sequence
Uba1	Ubiquitin-like modifier-activating enzyme 1
UbcD1 (eff)	Effete
upd	unpaired
USP36	Ubiquitin-Specific Protease 36
Utx	Utx histone demethylase
VEGF	Vascular endothelial growth factor
VNC	Ventral nerve cord
VWF	Von Willebrand Factor
Wg	Wingless
Wng	Wengen
µg	microgram
µl	microlitre
°C	Degrees celsius

Chapter 1

Introduction

1.1. *Drosophila melanogaster* as a model organism

The fruit fly, *Drosophila melanogaster*, has been used as a model organism for over a century, with the first use being documented in 1901 at Harvard University. However, it was Thomas Hunt Morgan in the early 20th century who used *Drosophila melanogaster* to confirm the chromosomal theory of inheritance and showed that genes were located on chromosomes. He went on to receive the Nobel Prize in Physiology or Medicine in 1933 for identifying and characterising the role of chromosomes in heredity. In the early years, the fruit fly was used prominently for genetic investigation, however more recently this underlying knowledge of genetic mechanism has been used to further study development, behaviour, innate immunity and human disease (Jennings, 2011; Pandey and Nichols, 2011). Many biological properties are conserved between mammals and *Drosophila*, and the fly has become a powerful tool to model innate immunity along with neurological and metabolic diseases including Parkinson's disease, obesity and diabetes (Alfa and Kim, 2016; Baker and Thummel, 2007; Trinh and Boulianne, 2013; Whitworth, 2011).

There are many technical advantages of using *Drosophila* in research; as a model organism in the laboratory they are easy and inexpensive to maintain, they have a short life cycle and can be genetically modified in many ways (Stocker and Gallant, 2008). The DNA of *Drosophila*, much like that of humans, is carried in chromosomal pairs, however, unlike humans they only have four pairs: a pair of sex chromosomes (XX in females and XY in males) along with three pairs of autosomes. This low number of chromosomes makes genetic analysis much easier compared to other organisms and therefore an extremely useful tool in scientific research, especially for genetic modifications (Hales et al., 2015). Interestingly, many of the genes and signalling pathways found in *Drosophila* are highly conserved in mammals, 75% of all human disease genes have related sequences in *Drosophila* (Bier, 2005). *Drosophila* have been used to study homologous genes that cause a broad spectrum of human disease following disruption, including developmental, neurological and metabolic disorders (Yamamoto et al., 2014).

1.1.1. *Drosophila* life cycle

Drosophila undergo four life cycle stages; egg, larva, pupa and adult. The adult female can lay between 750-1500 eggs in a lifetime, and once the eggs are fertilised, the embryo develops within the egg before hatching into larva. The larva grows through three instars until it pupates and undergoes metamorphosis into an adult fly (Figure 1-1). Most embryonic and larval tissues are lost during metamorphosis and the adult tissues develop from imaginal disc cells that are present from early

embryonic development (Klebes et al., 2002). The timing of the *Drosophila* life cycle is dependent on the temperature in which they are raised, at 25°C their development into adulthood takes around 10 days.

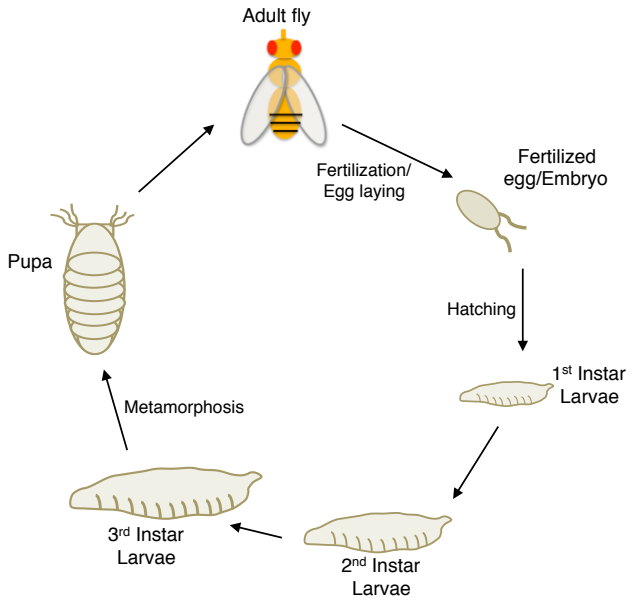


Figure 1-1: The *Drosophila* life cycle
Following mating, the female lays eggs and the embryos hatch giving rise to the 1st instar larvae. The larvae grow in two further instars (2nd and 3rd) until reaching pupal formation, and finally the adult's eclose.

Anatomically, *Drosophila* adults have an open circulatory system consisting of the hemolymph, a solution of water, inorganic salts and organic compounds that bathe the organs of the fly (Arrese and Soulages, 2010). They have a heart-like organ in the dorsal abdomen that consists of eight chambers in a tube-like structure, which are made up of pericardial cells and are closely surrounded by blood cells termed hemocytes (Grigorian and Hartenstein, 2013). The heart pumps the hemolymph through the organism and allows nutrients to distribute to all organs. They also have a respiratory system, trachea, a compartmentalised digestive system including the Malpighian tubules, which serve a similar function to the mammalian kidney, a liver-like organ known as the fat body and a brain that is used extensively to study neurological development, function and disease (Jeibmann and Paulus, 2009).

1.2. The *Drosophila* immune response

Throughout the last thirty years, *Drosophila* has become an increasingly important model organism for immunological research. Mechanisms of the immune response have been well conserved throughout evolution, and many molecular pathways, including those involved in immunity, can be studied in vertebrates, invertebrates and plants (Beutler, 2004). However, using *Drosophila* to study innate immunity provides

benefits due to the easily manipulated genome and the genetic tools available. In 2011, Jules Hoffmann was awarded the Nobel Prize in Physiology or Medicine, he used *Drosophila* as a model system to study the first line of defence against microbes and showed that the gene '*Toll*' was necessary for *Drosophila* to fight fungal infection (Lemaitre et al., 1996).

Insects, including *Drosophila*, have a complex immune system consisting of both cellular and humoral immunity. Cellular immunity consists of cells directly phagocytosing and killing invading pathogens or phagocytosing dead cells (Browne et al., 2013). The humoral immune response is able to secrete soluble molecules, such as antimicrobial peptides (AMPs), into the hemolymph to kill microbes that may not be in close proximity to the humoral response cells (Flatt et al., 2008a). The immune system of many organisms provides protection from a range of pathogens, many of which may be harmful to the host following infection. The ability for an individual to defend itself against pathogens and to distinguish between self and non-self is critical for survival (Akira et al., 2006). Studies in *Drosophila* and in mammals revealed highly conserved defence strategies at a molecular level (Lemaitre and Hoffmann, 2007).

Several different defence mechanisms have been shown to prevent microbes from harming the host (Figure 1-2). In many cases, the first line of defence includes physical barriers, including the exoskeleton or cuticle of insects as well as epithelial barriers of the trachea and gut (Reumer et al., 2010). If the first line of defence becomes breached, various specialised cells and tissues are responsible for the containment and eradication of the invading microbes. *Drosophila* lack an adaptive immune response, they rely solely on their innate immune system. Hemocytes are the cells that make up the cellular response in *Drosophila* by phagocytosing foreign intruders and dead cells, but also by providing encapsulation and melanisation responses in the fly (Vlisidou and Wood, 2015). The humoral response is characterised by the release of AMPs into the hemolymph, with the fat body being the major site of AMP production (Imler and Bulet, 2005).

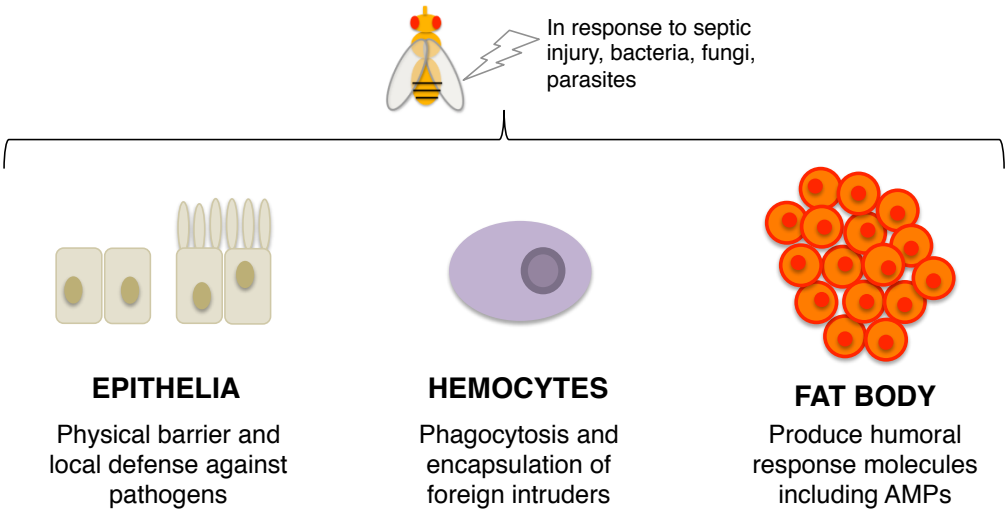


Figure 1-2: *Drosophila* immune defence mechanisms
Following septic injury or infection by bacteria, fungi or parasites, *Drosophila* can react by inducing the innate immune response via three main immune tissues; epithelia, hemocytes and the fat body.

1.2.1. *Drosophila* immune pathways

There are two major immune pathways characterised in *Drosophila*, the *Toll* pathway and the immune deficiency (*Imd*) pathway, which are the major pattern recognition pathways in the fly (Lemaitre and Hoffmann, 2007). The activation of these pathways are responsible for an upregulation of AMPs and cytokines in response to infection (Lemaitre and Hoffmann, 2007), and they control the majority of genes regulated by microbial infection (De Gregorio et al., 2002). The fat body, an organ analogous to the mammalian liver and adipose tissue, is the major site of the humoral immune response, being the main producer of AMPs following immune challenge (Imler and Bulet, 2005). Within these pathways, there are three *NF-κB*-like transcription factors that regulate *Toll* and *Imd* signalling, these are *Dorsal-related immunity factor* (*Dif*), *dorsal* and *Relish* (Dushay et al., 1996; Ip et al., 1993; Reichhart et al., 1993). It has been shown that *Dif* and *dorsal* are primarily associated with the *Toll* pathway, whereas *Relish* has been implemented in *Imd* pathway activation (De Gregorio et al., 2002). Both the *Toll* and *Imd* pathways are activated by peptidoglycan recognition proteins (PGRPs), which are ubiquitously expressed innate immunity molecules conserved from insects to vertebrates (Dziarski and Gupta, 2006). Insects have multiple PGRP genes, which are classified into short or long transcripts and are found in the hemolymph, cuticle, fat body and in the epidermal cells of the gut where they can generate AMPs, induce phagocytosis and hydrolyse peptidoglycan (Royet and Dziarski, 2007). Interestingly, Lemaitre et al (1997) showed that different infectious agents mount significantly different AMP expression profiles in response to infection.

This suggests the *Drosophila* immune response has the capability to differentiate between various invading microorganisms (Lemaitre et al., 1997).

The *Toll* pathway (Figure 1-3) shows evolutionarily conserved similarities to the vertebrate *interleukin 1 receptor* (IL-1R) signalling pathway (Gay and Keith, 1991). However, unlike the mammalian *Toll like receptors* (*TLRs*), *Toll* in *Drosophila* does not act as a pattern recognition receptor (PRR) itself and does not directly sense invading microbes (Janssens and Beyaert, 2003), it is activated by the cytokine *Spätzle* (*Spz*). *Toll* relies on three pathogen recognition proteins in the hemolymph that detect the invading pathogens and trigger a serine protease cascade that activates the *Spätzle*-processing enzyme (SPE), which in turn cleaves Spz into fragments (Stokes et al., 2015). The precursor form of Spz has no signalling activity, but the cleaved C-terminal region, which forms a cysteine knot structure, can activate the receptor (Weber et al., 2007). The pathway is activated by this cleavage event and the C-terminal of Spz can bind as a dimer to Toll, inducing dimerisation at the membrane, triggering the recruitment of three intracellular Death Domain-containing proteins, *Myeloid differentiation primary response gene 88* (*MyD88*), *tube* and the kinase *pelle*. *cactus*, the vertebrate *IκB* homolog, builds a complex with two *NF-κB* family members, Dorsal and Dif*,* in the cytoplasm and prevents them from translocating into the nucleus. Following the activation of Toll and the recruitment of MyD88, tube and pelle, cactus is phosphorylated, prompting ubiquitination and degradation by the proteasome (Daigneault et al., 2013). As a consequence of cactus degradation, Dif and Dorsal are released and translocate into the nucleus (Belvin and Anderson, 1996). They are then able to bind to *κB* binding sites and activate the transcription of AMP target genes including *Drosomycin (Drs)* (Imler and Bulet, 2005).

The *Imd* pathway (Figure 1-3) is activated directly via the binding of monomeric or polymeric diaminopimelic acid (DAP)-type peptidoglycan (PGN) of Gram-negative bacteria. The main transmembrane (type II) receptor of the *Imd* pathway known as peptidoglycan recognition protein-LC (PGRP-LC) recruits the death-domain containing adaptor Imd, in turn interacting with *Fas-associated death domain* (*FADD*), which binds the *Death related ced-3/Nedd2-like caspase* (*Dredd*) (Ferrandon et al., 2007). *Dredd* has been shown to associate with the *NF-κB* family member *Relish,* and is cleaved following Relish phosphorylation (Guntermann and Foley, 2011). Following cleavage, Relish translocates to the nucleus and binds to *κB* binding sites, whereas the inhibitory domain of Relish, known as the *ANKyrin repeats* (*ANK*), remains stable in the cytoplasm (Meister et al., 2005). The recruitment of Imd can also lead to Relish phosphorylation via the MAPKKK transforming growth factor-β (*TGFβ*)-activated kinase 1 (*TAK1*) and the *IKK* signalling complex formed by the

catalytic subunit *immune-response deficient 5* (*IRD5,* mammalian *IKKβ*) and a regulatory subunit known as *Kenny*, which is homologous to the mammalian *IKKγ* (Stokes et al., 2015). The translocation of *Relish* into the nucleus via FADD and Dredd or TAK1 leads to the activation of AMP target genes including *Attacin (AttA), Cecropin (CeCA), Diptericin (Dipt)* and *Drosocin (Dro)* (De Gregorio et al., 2002).

Negative regulation of the *Imd* pathway plays important roles in the restriction of signalling both outside and inside of cells and is critical for preventing unnecessary induction or hyperactivation of the immune response (Kim et al., 2006). Outside the cell, secreted catalytic PGRP proteins are able to reduce epithelial and systemic immune responses by scavenging peptidoglycan and PGRPs with amidase activity are able to down-regulate the *Imd* pathway following microbial sensing (Paredes et al., 2011). Within the cell, there are a number of negative regulators of the pathway including poor Imd response upon knock-in (pirk), which negatively regulates the PGRP-LC receptor (Kleino et al., 2008) whilst the *Drosophila* ubiquitin-specific protease, dUSP36, targets Imd itself to prevent constitutive immune signalling (Thevenon et al., 2009). Cylindromatosis (CYLD), a deubiquitinating enzyme, inhibits the IKK complex, which is made up of Kenny and IRD5 and defense repressor 1 (dnr1) supresses the activity of DREDD (Guntermann et al., 2009). The immune suppressor, *Caspar*, inhibits the Imd pathway by blocking DREDD-dependent cleavage and nuclear translocation of Relish (Kim et al., 2006). Relish itself plays a crucial role in limiting signalling through proteosomal degradation of TAK1 (Park et al., 2004). Furthermore, Trabid (trbd) a novel component of the Imd pathway is able to negatively regulate TAK1 (Fernando et al., 2014). Interestingly, mutations within intracellular regulators of the Imd pathway predispose flies to toxic levels of AMPs and compromised lifespan (Kounatidis et al., 2017).

Studies have also shown that the *Toll* and *Imd* pathways are not mutually exclusive, and the AMPs *Defensin (Def)* and *Metchnikowin (Mtk)* can be produced by either pathway (Tanji et al., 2007).

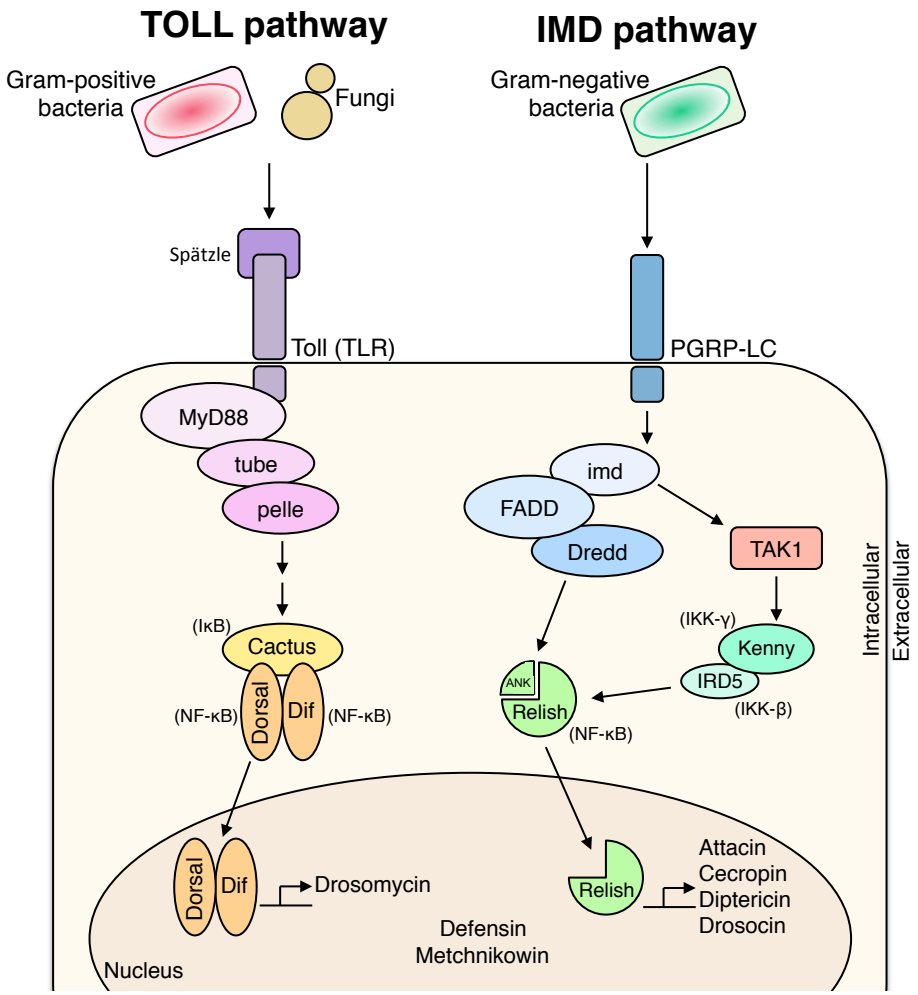


Figure 1-3: *Drosophila* Toll and *Imd* Pathways
Antimicrobial peptide (AMP) production is induced by two signalling cascades; the *Toll* and Immune deficiency (*Imd)* pathways. Left: *Toll* is activated by Gram-positive bacteria and fungi. The Toll receptor is activated upon binding of the cleaved form of Spätzle, inducing dimerisation at the membrane and the recruitment of three intracellular Death domain-containing proteins (*MyD88, tube, pelle*). *cactus*, the homolog of IκB, is phosphorylated and degraded by the proteasome. The transcription factors Dif and Dorsal are released, move into the nucleus and mediates the expression of antimicrobial peptides including *Drosomycin*. Right: PGRP-LC sense the presence of Gram-negative bacteria and activates the *Imd* pathway. Imd interacts with FADD and the caspase, Dredd. Dredd can then phosphorylate Relish, which is cleaved and Relish migrates to the nucleus to regulate expression of AMPs, while the ANK repeats remain in the cytoplasm. Relish can also be phosphorylated by the IKK signalling complex (Kenny and IRD5), which is activated through TAK1. The translocation of Relish to the nucleus mediates the expression of antimicrobial peptides including *Attacin, Cecropin, Diptericin* and *Drosocin*. The AMPs *Defensin* and *Metchnikowin* can be produced by both the *Toll* and *Imd* pathways.

1.2.2. *Drosophila* antimicrobial peptides (AMPs)

In *Drosophila,* 20 AMPs have been identified following immune induction that can be grouped into seven main classes of peptides; Attacin, Cecropin, Defensin, Diptericin, Drosocin, Drosomycin and Metchnikowin (Lemaitre and Hoffmann, 2007). These can

be further grouped into functional subgroups depending on their response to Gram-positive bacteria, Gram-negative bacteria or fungi. AMPs, a hallmark of the systemic humoral immune response, are small molecules that can exhibit a range of activities following the invasion of bacteria and fungi (Imler and Bulet, 2005; Meister et al., 1997). Elevated AMP expression levels can also be observed during times of stress, such as starvation or changes in temperature (Tsuzuki et al., 2012). AMPs are transcribed mainly in the fat body but also by epithelial cells and hemocytes (Ganesan et al., 2011). They are released into the hemolymph where they accumulate in high concentrations and circulate throughout the body (Lemaitre and Hoffmann, 2007). Insect AMPs, much like those of vertebrates, disrupt the integrity of membranes to kill the target microbes (Ganz, 2003).

1.2.3. Jak/STAT signalling in *Drosophila* innate immunity

The *Janus Kinase/Signal Transducer and Activator of Transcription* (*Jak/STAT*) pathway is another evolutionarily conserved pathway across many species, with functions ranging from development to the immune response. In *Drosophila*, the Jak/STAT pathway plays a number of key roles in development, including sex determination and embryonic segmentation (Zeidler and Perrimon, 2000). During larval development, Jak/STAT signalling is important for the development of both the eye and wing imaginal discs (Hombría and Sotillos, 2013) and is shown to be crucial for hematopoiesis, hemocyte development and differentiation in the larval lymph gland (Krzemien et al., 2010; Morin-Poulard et al., 2013). Additionally, Jak/STAT signalling has well-characterised roles in stem cell maintenance in the midgut and the maintenance of hematopoietic stem cells (HSCs) in the lymph gland (Bausek, 2013; Singh et al., 2005). Studies have also shown that Jak/STAT signalling plays an important role in immunity, especially in the immune response following viral infection (Agaisse and Perrimon, 2004; Myllymäki and Rämet, 2014). Along with the *Toll* and *Imd* pathways, the interplay between these three immune pathways has been shown to trigger the appropriate response to combat injury and infection (Dostert et al., 2005).

In *Drosophila,* the Jak/STAT pathway (Figure 1-4) consists of one Janus kinase, known as *hopscotch* (*hop*)*,* and one STAT transcription factor, known as *STAT92E* (Hou et al., 1996; Yan et al., 1996). There are three known ligands for the pathway, *unpaired* (*upd*), *unpaired 2* (*upd2*) and *unpaired 3* (*upd3*) that can function in either an autocrine or paracrine manner (Agaisse et al., 2003; Harrison et al., 1998; Hombría and Sotillos, 2013). Interestingly, the upd proteins show similarity to that of type I cytokines of the IL-6 family in vertebrates (Panayidou and Apidianakis, 2013).

The pathway becomes activated when upd, upd2 or upd3 binds to the gp130-like receptor, *domeless* (*dome*), which becomes dimerised and activates *hop* leading to the phosphorylation of the cytoplasmic domain of *dome* (Binari and Perrimon, 1994). This phosphorylation event allows the recruitment of STAT92E via its Src Homology 2 (SH2) domain, which can be recruited to *dome* and phosphorylated by *hop* (Hou et al., 1996). Upon phosphorylation, STAT92E dimerises and translocates as a homodimer to the nucleus, activating the transcription of its target genes, including *Turandot A* (*TotA)*, *thiolester-containing protein-1* (*TEP-1)* and *Suppressor of cytokine signalling 36E* (SOCS*36E)* (Yan et al., 1996).

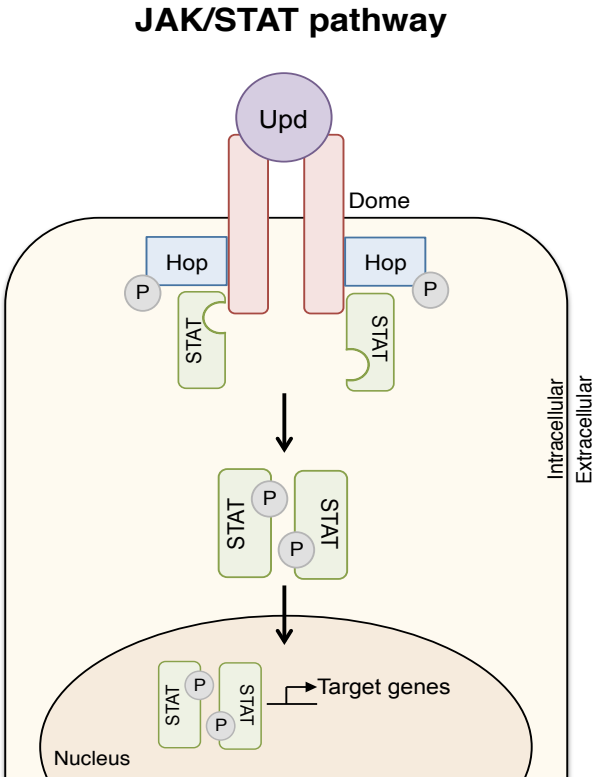


Figure 1-4: The Jak/STAT pathway in *Drosophila*
The unpaired ligands (upd1, 2, 3) bind and activate the receptor dome on the cell surface. This results in the activation of hop, leading to the phosphorylation of dome. The phosphorylated domain of the receptor becomes a docking site for STAT92E (STAT). STAT becomes phosphorylated, producing an active STAT dimer that moves into the nucleus and binds to DNA to activate the transcription of target genes. upd; unpaired, dome; domeless, hop; hopscotch, Jak; Janus kinase, STAT; signal transducers and activators of transcription.

1.2.4. Other pathways involved in the *Drosophila* immune response

In addition to the *Toll*, *Imd* and *Jak/STAT* pathways there are also a number of other pathways involved in *Drosophila* immune response including the JNK pathway, *p38* pathway and *TGF-β* signalling.

The Jun-N-terminal Kinase (JNK) is a member of the mitogen-activated protein (MAP) kinase family. In many organisms, the JNK pathway has been implicated in

numerous cellular responses including cellular proliferation, wound healing, apoptosis and inflammation (Weston and Davis, 2002). In *Drosophila,* there is a single *JNK* homolog, known as *basket* (*bsk*), and the pathway plays important roles in dorsal closure during embryonic development (Stronach and Perrimon, 2002) and for proper wound healing of the epidermis (Bosch et al., 2005). The *Drosophila* proteins eiger and wengen (wng) show similarities to mammalian *tumour necrosis factor* (*TNF*) and the *TNF receptor* (*TNF-R*), respectively (Moreno et al., 2002). *eiger*, the first invertebrate *TNF* superfamily ligand, is expressed predominantly in the nervous system and induces cell death by activating the JNK pathway through *wng* (Igaki et al., 2002). *eiger* has also been shown to trigger cell death and mediate pro-apoptotic function via *JNK* through *grindelwald* (*grnd*), another *TNF-R* family member (Andersen et al., 2015). Activation of both the JNK pathway and the *NF-κB* pathway, *Imd*, can be dependent on *Tak1*, which is homologous to mitogen-activated protein kinase kinase kinase (MAPKKK) in mammals. Interestingly, it has been shown that *Tak1* can activate the *JNK* pathway in response to bacterial infection, but the activation does not lead to the transcription of AMPs (Silverman et al., 2003). Activation of the JNK pathway also results in the activation of the transcription factor *activator protein-1* (*AP-1*) (Whitmarsh and Davis, 1996). *AP-1* plays important roles in controlling expression of genes including *Fos* and *Jun*, but also the expression of AMP genes may require the activation of *AP-1* function at the onset of immune response (Delaney et al., 2006) (Figure 1-5).

The *p38* MAPK pathway is an evolutionarily conserved stress response and has been shown to play roles in the immune response of both plants and animals (Hommes et al., 2003). The signalling of the *p38* pathway has also been described in *Drosophila* development, stress response and immunity. In *Drosophila,* there are three orthologues of *p38*, known as *p38a*, *p38b* and *p38c* (Chen et al., 2010), which have been shown to have various functional roles. *Drosophila* lacking the *p38a* gene are susceptible to environmental stresses such as starvation and heat shock (Craig et al., 2004). Whereas, *p38b* is involved in *decapentaplegic* (*dpp*) signal transduction during wing morphogenesis (Adachi-Yamada et al., 1999) and *p38c* is expressed in the gut, upregulated during intestinal infection and regulates lipid metabolism and immune homeostasis in the intestine (Chakrabarti et al., 2014). In response to stress, *Drosophila* p38 can directly phosphorylate *Activating transcription factor-2* (*Atf-2*), a major transcriptional activator of stress-inducible genes that is critical for *p38*-mediated stress response (Sano et al., 2005) (Figure 1-5).

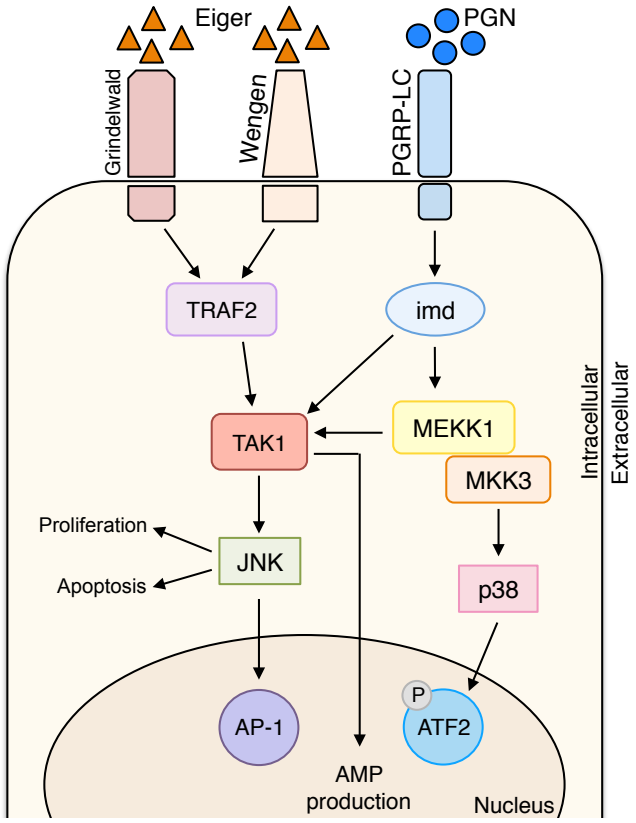


Figure 1-5: The JNK and p38 pathway in *Drosophila*
The conserved JNK pathway is activated by *eiger*, the TNF-superfamily ligand, which signals through two receptors, grindelwald and wengen. eiger binds to the receptors and activates TRAF2, which in turn activates JNK. The activation of *JNK* leads to the phosphorylation and activation of transcription factors including *AP-1*. The p38 pathway can be activated by the PGN of invading pathogens, which are sensed by PGRP-LC, triggering the Imd and p38 mitogen-activated protein kinase pathways. The activation of p38 via the MEKK1/MKK3 complex enhances the transactivating function of ATF2. PGN; peptidoglycan, PGRP-LC; peptidoglycan-recognition protein LC, TRAF2; tumour necrosis factor receptor-associated factors, Imd; immune deficiency, TAK1; TGF-β activated kinase 1, MKK3; MAP kinase kinase 3, JNK; Jun amino-terminal kinase, AP-1; activator protein-1, ATF2; activating transcription factor 2, AMP; antimicrobial peptide.

The transforming growth factor-beta (*TGF-β*) superfamily are a large group of proteins that regulate many things including development and the immune response in both vertebrates and invertebrates (Raftery and Sutherland, 1999). Ligands of this superfamily signal through a type I/II receptor complex resulting in the phosphorylation and activation of specific *SMAD* transcription factors (Derynck and Zhang, 2003). *SMAD* transcription factors form complexes with Co-*SMADs* in the cytoplasm upon phosphorylation and translocate to the nucleus. Here, they can bind directly to DNA and activate or repress transcription to regulate target gene expression (Attisano and Tuen Lee-Hoeflich, 2001). In *Drosophila,* the *TGF-β* signalling pathway comprises of two main groups, the activin group and the bone morphogenetic protein (BMP) group. The activin subfamily, has three ligands, *Activin-*

beta (*Actβ*), *dawdle* (*daw*) and *myoglianin* (*myo*), which all signal through the single *baboon* (*babo*) receptor (Ghosh and O'Connor, 2014). Conversely, the BMP group also consists of three different ligands, *dpp*, *glass-bottom boat* (*gbb*) and *screw* (*scw*), which can signal through two receptors known as *thick veins* (*tkv*) and *saxophone* (*sax*) (Nguyen et al., 1998). Both the activin and BMP groups can regulate different aspects of the innate immune response, for example *dpp* and *daw* both respond to immune challenge. However, *dpp* expression is increased during sterile injury and supresses unnecessary AMP expression, whereas *daw* expression is increased in response to infection and is an important inhibitor of inflammation following injury (Clark et al., 2011).

1.3. The fat body

The insect fat body plays many important roles in the humoral immune response, fat and glycogen storage, lipid metabolism and nutrient sensing (Arrese and Soulages, 2010). Fat body tissue originates from the mesoderm during embryogenesis (Hoshizaki et al., 1994) and becomes immune competent in the early larval stage (Lemaitre and Hoffmann, 2007). The larval fat body is the main energy store during pupal development and during the first few days of adulthood following eclosion. After the first days of adulthood, the freely floating cells of the larval fat body undergo apoptosis and are replaced by newly developing adult fat body cells (Liu et al., 2009). The new fat body cells develop in the adult *Drosophila* and perform a similar energy storing function. In adult *Drosophila,* the fat body is distributed throughout the body, located mainly under the exoskeleton and surrounding the gut and reproductive organs (Arrese and Soulages, 2010). Structurally, the tissue is arranged in thin lobes, which allows maximal exposure to the hemolymph and rapid detection of changes in energy levels or circulating hormone levels (Arrese and Soulages, 2010).

1.3.1. The fat body in metabolism

The fat body is not only the main humoral immune response organ; it is also the primary metabolic tissue in *Drosophila*. Fat body cells are responsible for the synthesis and utilisation of lipids, carbohydrates and amino acids, as well as the synthesis of almost all the hemolymph proteins and metabolites (Arrese and Soulages, 2010). In recent years, the link between immunity and metabolism has been explored, with infections prompting metabolic dysregulation and dysfunction. *Mef2*, a transcription factor originally characterised for its role in muscle development, is required in the fat body for anabolic function and immune response, and is a critical

transcriptional switch between metabolism and immunity in the adult fat body (Clark et al., 2013).

Insulin/insulin-like growth factor (IGF) signalling (IIS) and target of rapamycin (TOR) signalling pathways (Figure 1-6) are central to metabolic regulation and controlling the uptake of nutrients into cells of *Drosophila.* Both these pathways can function either independently or together in an insulin/Akt/TOR-signalling network.

The insulin-signalling pathway has been shown to play roles in regulating lifespan, growth and metabolism via the insulin-like receptor (*InR*) (Goberdhan and Wilson, 2003). Following initiation of the insulin pathway by *Drosophila* insulin-like peptides (DILPs), *chico*, the insulin receptor substrate becomes activated leading to the phosphorylation of the pathway antagonist *Akt* (also known as protein kinase B) (Scanga et al., 2000). This phosphorylation event leads to the phosphorylation of the forkhead transcription factor FOXO leaving it inactive in the cytoplasm (Hay, 2011). When FOXO is not phosphorylated, it is able to move into the nucleus and activate the transcription of various genes including 4E-binding protein (*4E-BP,* also known as *Thor* in *Drosophila*), a known inhibitor of protein translation (Webb and Brunet, 2014). Insulin signalling controls a wide range of metabolic and physiological processes in the cell, ranging from autophagy to nutrient sequestration (Britton et al., 2002; Kim and Neufeld, 2015). Mutations in the insulin pathway can be detrimental to *Drosophila*, mutations in *chico* severely disrupts cellular proliferation and growth (Naganos et al., 2012) and insulin pathway mutant flies are significantly smaller, with higher lipid content compared to control flies (Murillo-Maldonado et al., 2011).

TOR is a highly conserved serine/threonine kinase that is involved in regulating cell growth, stress levels and metabolism (Inoki et al., 2005). The TOR pathway can be activated by insulin signalling, via the InR and AKT, or by sensing intracellular amino acids or various extracellular nutrients such as amino acid in the hemolymph (Miyamoto et al., 2013). Activated *AKT* is able to suppress the activity of the tuberous sclerosis complex genes 1 and 2 (*TSC1/TSC2*) complex, which inactivates the Ras homologue enriched in brain (*Rheb*) and results in TOR activation. *Rheb* promotes cell growth in a TOR and S6 Kinase (S6K)-dependent manner (Saucedo et al., 2003; Stocker et al., 2003). The activity of S6K, a p70 ribosomal protein, also leads to an increase in protein synthesis and cellular proliferation (Volarević and Thomas, 2001). However, *Drosophila* lacking the *S6K* gene show extreme delays in development and a severe reduction in body size, which is due to the flies having smaller cells, not fewer cells (Montagne et al., 1999). Whether TOR activation is insulin-dependent or via nutrient sensing, TOR phosphorylates targets such as *S6K* and *4E-BP* to initiate translation (Miron et al., 2003). TOR mutant flies

show an increase in the lipid metabolism gene, *brummer*, and a decrease in *fatty acid synthase* (*FAS*) transcript levels, suggesting the activity of TOR regulates a balance between lipid anabolism and catabolism (Birse et al., 2010).

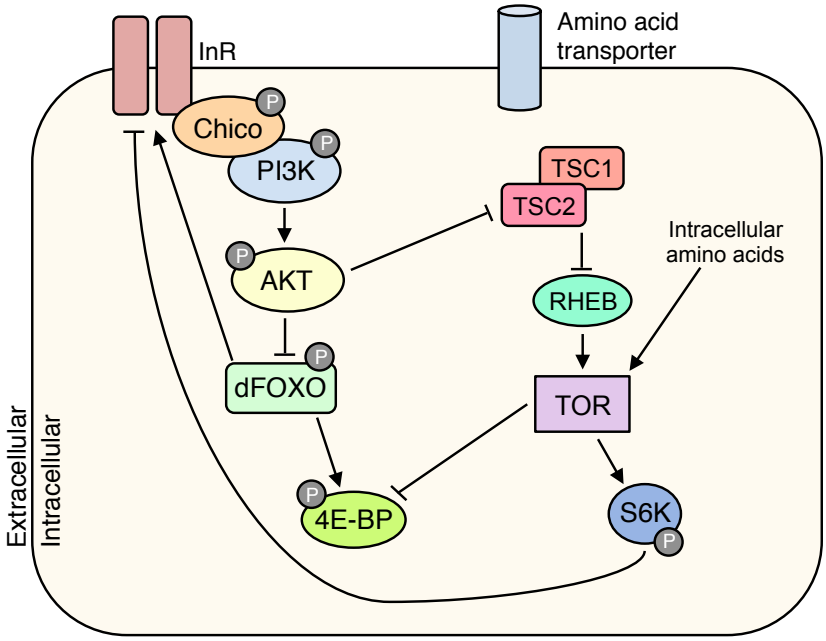


Figure 1-6: Insulin and TOR signalling in *Drosophila*
The InR is activated by the binding of DILPs. Following activation, the InR transduces a signal from the DILPs to PI3K, through the single *Drosophila* insulin receptor substrate, chico. The major kinase AKT becomes phosphorylated and activated, which subsequently phosphorylates the transcription factor FOXO, leaving it inactive in the cytoplasm. The TOR pathway plays an important role in controlling amino acid sensing via amino acid transporters. Following the phosphorylation of TOR, the TOR kinase is able to activate S6 kinase and inhibit the activity of 4E-BP. The insulin-signalling pathway and the TOR pathway interact at the level of AKT, the activation of AKT leads to the inhibition of the TSC1/2 complex and the activation of the TOR pathway. DILPs; *Drosophila* insulin-like peptides, InR; Insulin-like receptor, PI3K; Phosphoinositide 3-kinase, 4E-BP; 4E-binding protein, TSC1/TSC2; tuberous sclerosis complex genes 1 and 2, RHEB; Ras homologue enriched in brain, TOR; target of rapamycin, S6K; S6 kinase.

1.3.2. Lipid and sugar metabolism in *Drosophila*

Drosophila share most of the basic metabolic functions found in vertebrates, they are able to maintain energy stores in the form of lipids and glycogen as well as uphold the appropriate level of circulating sugars, trehalose and glucose (Rajan and Perrimon, 2013).

Lipids have an important function in the composition of cellular membranes and in energy storage. In *Drosophila*, lipids in the form of triacylglycerol (triglycerides or TAG) and cholesterol ester are synthesised from dietary carbohydrates, fatty acids or proteins and are stored in the fat body as lipid droplets (LDs). The mobilisation of lipids is essential for growth, reproduction and energy requirements during times of non-feeding (Arrese and Soulages, 2010) and a balance between TAG storage and breakdown is crucial for the maintenance of energy homeostasis in both vertebrates and invertebrates (Trinh and Boulianne, 2013). In times of energy requirement, TAG stores are accessed by a number of lipases, which help orchestrate the breakdown of lipids via lipolysis (Figure 1-7). *brummer* (*bmm*), a homologue to mammalian *adipocyte triglyceride lipase* (*ATGL*), is an important lipid storage droplet-associated TAG lipase (Grönke et al., 2005). Studies show that during starvation or an over-expression of *bmm* in *Drosophila* depletes lipid stores, whereas a loss of *bmm* can cause obesity in flies (Grönke et al., 2005). *Hormone sensitive lipase* (*Hsl*) mutant larvae have significantly larger LDs following starvation, however adult *Hsl* mutants have only slightly increased TAG stores than controls in fed conditions (Lee et al., 2013). Additionally, in mammals *ATGL* and *Hsl* are thought to work in a synergistic manner to accomplish lipolysis at the LD surface (Grönke et al., 2007). *Drosophila perilipin 1* and *perilipin 2* (*plin1* and *plin2* or *lipid storage droplet 1* and *2*; *Lsd1*, *Lsd2*) associate with lipids, more specifically *plin1* generally labels LDs of varying size and interacts closely with *Hsl* at the LD membrane to initiate the breakdown of LDs and begin the lipolysis of stored TAG. However, *plin2* localises to smaller LDs, is involved in promoting lipid accumulation and acts as a barrier to lipases such as *bmm* and *Hsl* (Bi et al., 2012). Interestingly, *plin1* locates exclusively to the surface of LDs while *plin2* can be present in both the cytoplasm and on the LD surface (Beller et al., 2010).

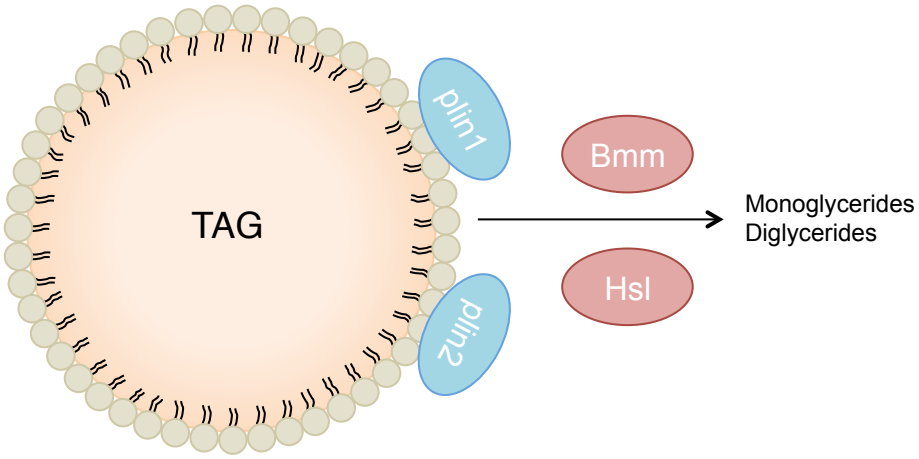


Figure 1-7: Lipases involved in *Drosophila* lipid metabolism
Stored lipid droplets become metabolically accessible by lipolysis; triglycerides are hydrolysed to monoglycerides and diglycerides. Lipases are shown in red and lipid droplet-associated proteins in blue. TAG; triacylglycerol/triglyceride, plin1; perilipin 1, plin2; perilipin 2, bmm; brummer, Hsl; Hormone sensitive lipase.

The glucagon-like adipokinetic hormone (Akh) family plays significant roles in stimulating the fat body to convert stored TAG into diglyceride and glycogen into trehalose, the principle sugar in insects (Matsuda et al., 2015). Trehalose is synthesised from glucose-6-phosphate synthase in the fat body of *Drosophila* and degraded back to glucose by the enzyme trehalase (Yasugi et al., 2017). Glucose from the diet can be stored as the carbohydrate glycogen in the fat body when it is not required for energy. Glycogen also provides a stored source of glucose for trehalose synthesis and the mobilisation of trehalose to glucose has been found to be critical for metabolic homeostasis (Yasugi et al., 2017). Interestingly, trehalose and glucose, the two circulating sugars in *Drosophila,* have been shown to play a direct role in the release of Akh by clusters of endocrine cells in the ring gland of the brain known as corpora cardiaca (Kim and Neufeld, 2015; Kim and Rulifson, 2004).

In addition to the fat body, hepatocyte-like cells known as oenocytes can enable lipid metabolism (Gutierrez et al., 2007) and express many lipid-synthesising and catabolising enzymes including *Fas* and *acetyl-CoA carboxylase* (*Acc*) (Parvy et al., 2012). In *Drosophila*, oenocytes are derived from ectoderm in two separate generations, one larval and one adult, with differing progenitors that are distinct with regards to cell number and size (Makki et al., 2014). During the larval stage oenocytes are much larger in size, however these cells breakdown and disappear before adult eclosion (Johnson and Butterworth, 1985). In adults, these cells are arranged in clusters in the abdominal segment and play important roles in development, growth and feeding behaviour (Gutierrez et al., 2007). Figure 1-8 shows

the metabolic homeostatic mechanism of the fat body and oenocytes in *Drosophila* larvae.

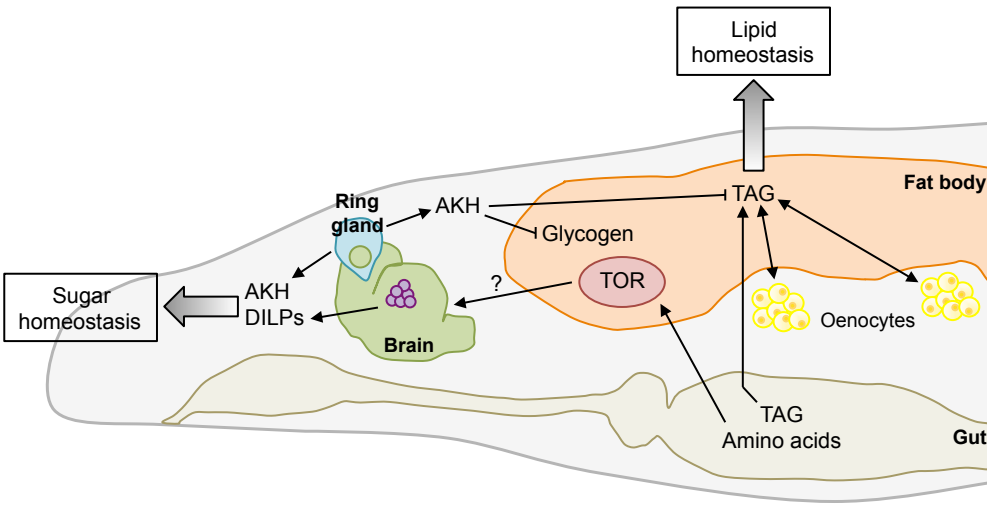


Figure 1-8: Metabolic homeostasis in *Drosophila* larvae
Sugar homeostasis is maintained by IPCs in the brain and the ring gland, producing DILPs and Akh. Akh release from the ring gland can inhibit the storage of glycogen and TAG in the fat body to maintain lipid homeostasis. Upon starvation, the fat body releases lipids that are taken up by oenocytes for the production of energy. Amino acids and TAG in the gut can activate the TOR pathway and TAG storage in the fat body, respectively. IPC; insulin producing cells, DILPs; *Drosophila* insulin-like peptides, Akh; Adipokinetic hormone, TOR; target of rapamycin, TAG; triacylglycerol/triglycerides (Adapted from Leopold and Perrimon, 2007).

1.3.3. Nutrient sensing by the *Drosophila* fat body

Nutrition is a key regulator in developmental timing in both vertebrates and invertebrates (Layalle et al., 2008). As discussed above, nutrients, such as lipids and glucose, are detected and stored in the form of TAG and glycogen.

In the fat body of *Drosophila*, insulin signalling and TOR signalling play important roles in maintaining nutritional homeostasis, and as a result can regulate nutrient uptake, metabolism and storage (Zhang and Xi, 2014). The insulin-signalling pathway contributes to energy homeostasis by regulating carbohydrate storage (Slack et al., 2010). Insulin signalling is also the principle regulator of lipid accumulation (DiAngelo and Birnbaum, 2009). TOR signalling can also be activated in the fat body by free amino acids (Kim, 2009), amino acid transporters, such as *slimfast*, can function as nutrient sensors and regulate endocrine activity of the fat body via TOR (Reynolds et al., 2007). TOR also negatively regulates cell autonomous mechanisms such as autophagy, which can provide amino acids during conditions of limited nutrients (Jung et al., 2010). Interestingly, reducing *InR* or phosphoinositide 3-kinase (*PI3K*), an important family of signal transducer enzymes, in the fat body decreases growth rate whereas decreasing TOR activity in the fat body causes a reduction in final body size (Britton et al., 2002; Colombani et al., 2003). In the *Drosophila* larval

fat body, the steroid hormone 20-hydroxyedysone (20E, ecdysone) integrates with the insulin-signalling pathway to monitor nutritional status. 20E in the fat body has been shown to antagonise the insulin-signalling pathway, promote autophagy (Rusten et al., 2004) and weaken *Myc* activity to suppress peripheral insulin signalling and body growth (Colombani et al., 2005; Delanoue et al., 2010). Interestingly, 20E also controls the length of the larval stages and developmental transitions (Nijhout et al., 2014).

The fat body is the major site for nutrient storage and mobilisation of lipids. Therefore, these processes have to be tightly regulated and communicated to other organs in larvae and adult flies to help maintain nutrient uptake and energy levels. One class of hormone peptides regulating these processes are the *Drosophila* insulin-like peptides (DILPs). In *Drosophila,* there are eight of these peptides (DILP1-8) with varying expression patterns and function. DILP secretion can regulate energy balance and promote growth in response to dietary sugars and fats. In recent years, more and more studies implicate a major role for the fat body in communicating the nutrient status to the insulin producing cells (IPCs) in the brain and other organs via the release of hormonal and cytokine signals (Zhang and Xi, 2014). During both larval and adult stages, DILP6 is expressed extensively in the fat body and is induced by 20E. In adult *Drosophila, DILP6* transcript levels are regulated by FOXO and its expression represses *DILP2* and *DILP5* mRNA in the brain and the secretion of DILP2 into the hemolymph (Bai et al., 2012). The production of DILP2, 3 and 5 by the brain have also been shown to cross-talk with DILP6 in the fat body and removing DILP2, 3 and 5 increases the expression of *DILP6* in the fat body.

The unpaired (upd) family are also thought to have important roles in nutrient sensing, along with their role in Jak/STAT activation in *Drosophila*. upd2, a functional analogue of leptin, is produced by the fat body and secreted into the hemolymph in response to dietary fat and sugar. upd2 activates the Jak/STAT pathway in GABAergic neurons, reducing their inhibitory effect on IPCs and in turn prompting the secretion of DILPs into the hemolymph (Rajan and Perrimon, 2012). This release of DILPs promotes uptake of nutrients, which results in growth and fat storage. Moreover, knockdown of *upd2* in the fat body reduces adult body size by preventing DILP2 release from IPCs (Zhang and Xi, 2014). upd2 has been considered to serve a similar function to mammalian leptin, however, *upd2* null flies do not show an obese phenotype (Rajan and Perrimon, 2012), whereas leptin deficient mammals show an increase in both fat storage and food intake (Friedman and Halaas, 1998). Recent studies have also shown *upd1*, another homolog of mammalian leptin, expression in the *Drosophila* brain but not fat body, increases food intake, weight and food cue

attraction (Beshel et al., 2017). Furthermore, the inflammatory cytokine *upd3*, which is released from hemocytes, specifically plasmatocytes upon excess of dietary lipids, was recently shown to play a detrimental role in reducing insulin sensitivity in various tissues (Agaisse et al., 2003; Woodcock et al., 2015).

Ecdysone-inducible gene L2 (*ImpL2*), the secreted insulin/IGF antagonist, is able to form protein complexes with DILP2 and DILP5 and inhibit insulin signalling in adult *Drosophila* (Alic et al., 2011). It is required for larval survival (Honegger et al., 2008) and in times of nutritional stress is upregulated in the fat body, which leads to the suppression of insulin signalling and allows *Drosophila* to endure periods of starvation. Kwon et al (2015) and Figueroa-Clarevaga & Bilder (2015) also showed that ImpL2 is a critical factor involved in systemic organ wasting in *Drosophila.*

There is also another group of peptides that are known to regulate nutrient sensing in adult *Drosophila*. Firstly, *Growth-blocking peptide 1* and *2* (*Gbp1*, *Gbp2*) are fat body-derived signals, synthesised in response to amino acids and the activation of TOR signalling. Both *Gbp1* and *Gbp2* stimulate DILP secretion from the brain and increase insulin signalling activity in *Drosophila* (Koyama and Mirth, 2016). Reducing expression of *Gbp1* and *Gbp2* in the fat body leads to reduced growth rate and a smaller body size. *Gbp1* specifically has been further shown to regulate the immune response and the expression of *Gbp1* is thought to be sensitive to starvation stress (Fujikawa et al., 2009). Secondly, the peptide hormone *CCHamide2* (*CCHa2*) is expressed in the fat body and gut, binds to its receptor (*CCHa2-R*) in the brain and acts as a nutrient-dependent regulator of DILPs in *Drosophila* (Sano *et al.,* 2015). Data has shown that CCHa2 and CCHa2-R form a direct signalling response between the brain and peripheral tissues (Sano, 2015). Moreover, transcriptional levels of *CCHa2* are altered with changing nutrient levels, particularly sugars and genetically disrupting either *CCHa2* or *CCHa2-R* causes defects in both larval growth and developmental timing (Sano et al., 2015).

Signalling from peripheral tissues, such as the fat body, to the brain is essential for regulating the growth and metabolism of *Drosophila* in response to nutritional availability. Mechanisms of nutrient sensing under normal physiological conditions, particularly by the fat body of *Drosophila* are shown in Figure 1-9.

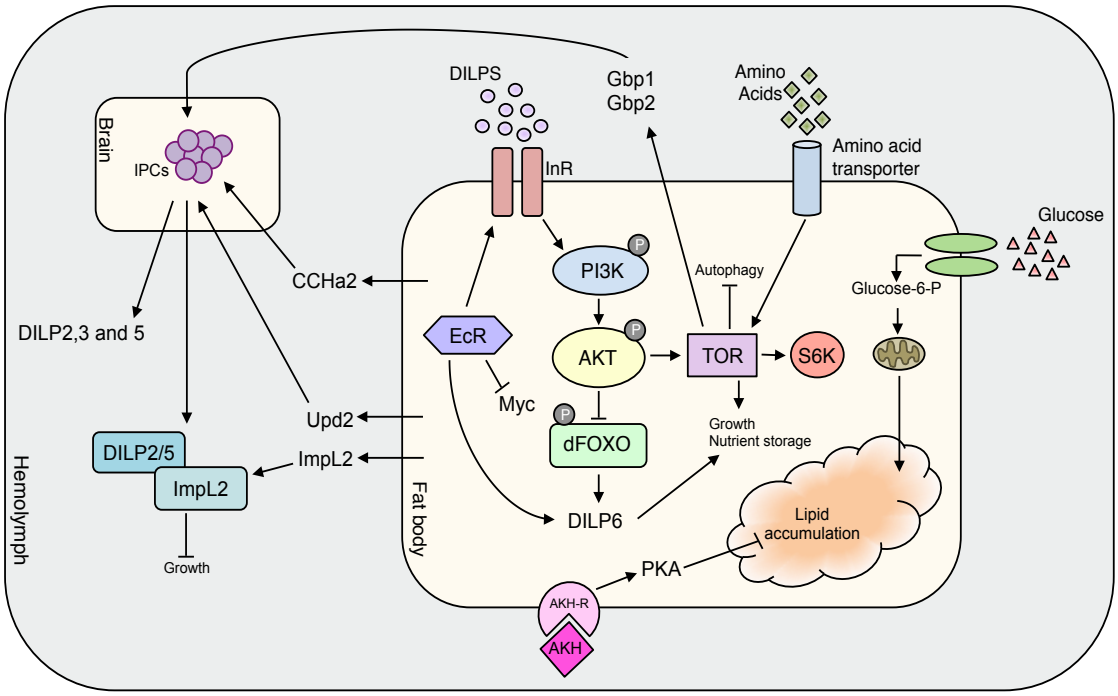


Figure 1-9: Nutrient-sensing mechanisms in *Drosophila*
Drosophila nutrient sensing pathways carried out between the brain, fat body and hemolymph. IPCs; insulin producing cells, DILP; *Drosophila* insulin-like peptide, Gbp; Growth-blocking peptide, InR; Insulin-like receptor, PI3K; Phosphoinositide 3-kinase, EcR; ecdysone receptor, TOR; target of rapamycin, S6K; S6 kinase, Akh; Adipokinetic hormone, AKH-R; adipokinetic hormone receptor, PKA; cAMP-dependent protein kinase, Glucose-6-P; glucose-6-phosphate, upd2; unpaired 2, CCHa2; CCHamide 2, ImpL2; Ecdysone-inducible gene L2.

1.3.4. Regulation of metabolism in fed and starved states

Regulating metabolism under physiological conditions is important to maintain the homeostasis of many organisms. During a stress response or environmental change, such as starvation, specific mechanisms are required to help aid survival (Schwasinger-Schmidt et al., 2012). *Drosophila* can regulate their metabolic pathways to trigger an accurate response in fed or starved states.

In the fed state, *Drosophila* IPCs in the brain release DILPs into the hemolymph. IPCs sense systemic glucose levels in the hemolymph in a cell autonomous manner and an uptake of glucose results in a depolarisation event in the IPCs. This depolarisation event activates voltage-sensitive calcium channels and triggers the release of DILPs (Nässel and Vanden Broeck, 2016). The circulating DILPs can then bind to the InR inducing downstream phosphorylation of AKT, in turn this can lead to the phosphorylation of FOXO and the activation of TOR signalling. FOXO phosphorylation prevents its movement from the cytoplasm to the nucleus therefore promoting growth and nutrient storage in the fat body. Nutrient signals, particularly cellular levels of amino acids, and insulin signalling via the InR can promote TOR activation. *Melted*, a modulator of the insulin-signalling pathway, is

found on the membrane of cells and can interact with both TSC1 of the TSC1/2 complex upstream of TOR and FOXO to recruit proteins to the cell membrane (Teleman et al., 2005a). *Melted* mutants have reduced lipids, a reduction in TOR signalling and an increase in *foxo* activation (Reis and Hariharan, 2005). *Slimfast*, an amino acid transporter, signals through TOR to regulate metabolism and growth in response to amino acid levels, it can also override insulin signalling in peripheral tissues by inhibiting PI3K activity (Baker and Thummel, 2007). The TOR pathway is made up of two multiprotein complexes: TORC1 and TORC2. TORC1 regulates growth and translation via the phosphorylation of S6K to drive protein synthesis or inhibit *4E-BP* activity to increase translation (Bjedov et al., 2010). Under favourable conditions, such as an amino acid-rich fed state, TORC1 is active and S6K can work as a positive mediator of the TOR pathway to promote growth and alter metabolism (Montagne et al., 1999; Um et al., 2006). TORC1-dependent phosphorylation of 4E-BP disrupts its association with translation initiation factor 4E (eIF4E) allowing eIF4E to promote cap-dependent translation (Richter and Sonenberg, 2005). Additionally in the fed state, fatty acids are rapidly taken up by the fat body and incorporated into glycerides, including TAG and phospholipids. Following feeding, TAG, glycogen and protein granules occupy most of the intracellular space within the fat body (Arrese and Soulages, 2010).

Upon starvation, DILP expression levels can vary dramatically in response to the reduction in nutrients available. Studies have shown levels of DILP3 and DILP5, but not DILP2, are reduced in starved *Drosophila* (Ikeya et al., 2002). In contrast, *DILP6* transcript levels are increased under nutritional deprivation and FOXO has been shown to modulate this response in larvae (Slaidina et al., 2009). Interestingly, an increased accumulation of DILP2 and DILP5 is shown in the IPCs of the *Drosophila* brain following starvation, whereas *DILP2* transcript levels in the whole fly remain unchanged (Kannan and Fridell, 2013). During the starvation state, the activity of both the insulin and TOR signalling pathways is reduced. Without the phosphorylation of FOXO by the insulin pathway, it is able to freely translocate into the nucleus, induce transcription of starvation response genes, reduce protein synthesis and restrict *Drosophila* growth (Baker and Thummel, 2007). A reduction in TOR activity following starvation leads to a reduction in S6K activity and a decreased ability to carry out translation via *4E-BP* (Tettweiler et al., 2005). The activity of 4E-BP, which can be controlled by TOR, has been shown to work as a metabolic brake that is activated under conditions of environmental stress, including starvation, to control fat metabolism (Teleman et al., 2005b). Adipokinetic hormone (Akh), which plays a similar role to mammalian glucagon, binds to the Akh-receptor (Akh-R) and

promotes the breakdown of TAG and glycogen in the fat body during starvation. Akh also activates the enzyme glycogen phosphorylase, which reduces the amount of glycogen in the fat body and increases circulating sugar levels (Baker and Thummel, 2007). During periods of starvation, the lipase *bmm,* which acts in parallel with Akh to regulate lipolysis, plays a crucial role in the mobilisation of TAG and glycogen in the fat body to promote energy release (Grönke et al., 2005). The fat body signalling pathways in both the fed and starved states are shown in Figure 1-10. Additionally, *Drosophila* oenocytes are thought to be critical to mount an effective starvation response, they are able to promote lipid and carbohydrate breakdown following fat body-derived DILP6 signalling and increase tolerance to starvation (Chatterjee et al., 2014).

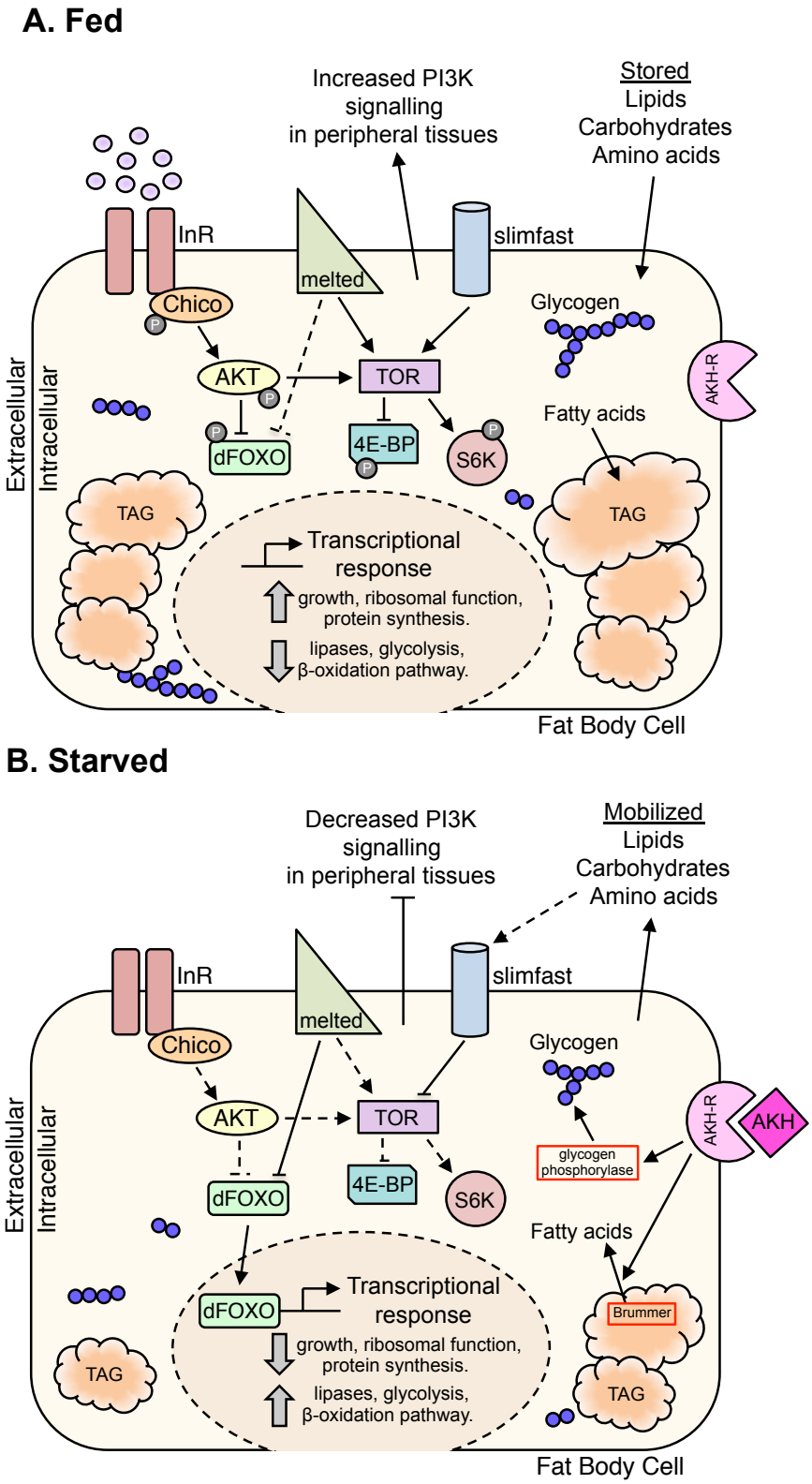


Figure 1-10: Metabolic pathways in fed and starved states

A. *Drosophila* metabolic pathways in the fat body in a fed state and **B.** *Drosophila* metabolic pathways in the fat body in the starved state. Solid lines represent active signalling pathways, and dotted lines represent regulatory mechanisms that become active when signalling is not active. InR; Insulin-like receptor, TAG; triacylglycerol/triglyceride, TOR; Target of rapamycin, S6K; S6 Kinase, Akh; Adipokinetic hormone, Akh-R; Adipokinetic hormone receptor, PI3K; phosphoinosiride-3-kinase (Adapted from Baker and Thummel, 2007).

1.4. Hemocytes

Hemocytes, the collective term for the blood cells of *Drosophila,* originate from mesodermally derived stem cells (Lavine and Strand, 2002) and can differentiate into three main blood cell types, plasmatocytes, crystal cells and lamellocytes, which function in embryonic, larval and adult *Drosophila*.

Plasmatocytes make up ~95% of circulating hemocytes in the adult fly and are most similar to mammalian leukocytes, particularly the monocyte and macrophage lineage (Holz et al., 2003). In *Drosophila* larvae, hemocytes circulate throughout the body, however in adults, much like mammalian macrophages, plasmatocytes are sessile cells and become resident cell populations following terminal differentiation (Elrod-Erickson et al., 2000). They have been shown to play important roles as professional phagocytes and in cytokine production (Agaisse et al., 2003; Charroux and Royet, 2009). Interestingly, adult plasmatocytes are not a homogenous population of cells as they exhibit differential expression of signalling molecules including *dpp* and *daw* in response to wounding and infection (Clark et al., 2011). Furthermore, adult plasmatocytes seem to be post-mitotic, and no cellular proliferation has been seen under steady state or in inflammatory conditions (Honti et al., 2014). Crystal cells contain crystalline inclusions of prophenoloxidase that function in the melanisation immune response of *Drosophila* (Gajewski et al., 2007), and play important roles in wound healing and encapsulation. The production of melanin also leads to the production of cytotoxic free radicals that may participate in the killing of invading pathogens (Meister and Lagueux, 2003). In larvae, lamellocytes are stress-response cells, which do not usually appear in healthy individuals (Avet-Rochex et al., 2010). They are able to specifically differentiate and encapsulate foreign bodies that are too large to be phagocytosed by plasmatocytes (Wood and Jacinto, 2007), such as the parasitoid wasp eggs laid in *Drosophila* larvae by the parasitoid wasp. Lamellocytes have not been yet been detected in adult *Drosophila.*

1.4.1. Hematopoiesis in *Drosophila*

Embryonic hematopoiesis in *Drosophila* is thought to be reminiscent of early hematopoiesis in vertebrates (Figure 1-11). During *Drosophila* development, two hematopoietic waves have been described: embryonic and lymph gland hematopoiesis (Evans et al., 2003). The embryonic and larval stages of hematopoiesis lead to the formation and expansion of self-renewing tissue-resident macrophages that emerge from the embryonic head mesoderm around embryonic stage 7 (Gold and Brückner, 2014). These new tissue-resident macrophages can colonise local microenvironments in peripheral tissues and can self-renew in the

differentiated state (Makhijani et al., 2011). The majority of these cells migrate throughout the larvae and a small number remain localised around the anterior part of the gut known as the proventriculus (Wood and Jacinto, 2007).

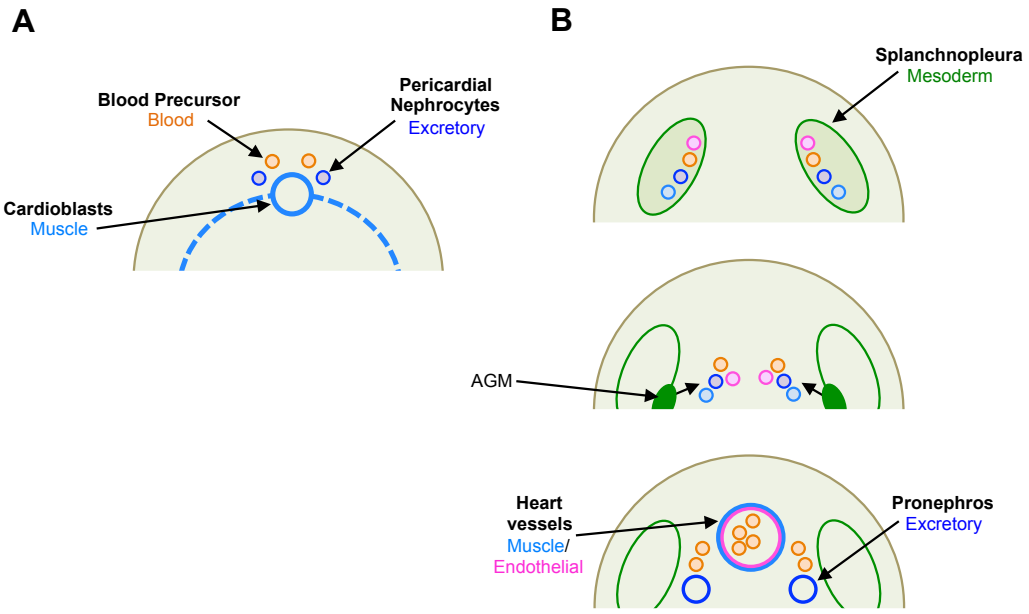


Figure 1-11: Early hematopoiesis in *Drosophila* and vertebrates
A. Insect blood, vascular and excretory cells derived from a common precursor. During development many different cell types stay in close contact, and the lymph gland and pericardial nephrocytes line up next to the dorsal vessel. **B.** In vertebrates, the AGM gives rise to endothelial and blood precursors that migrate within the embryo to assemble blood vessels and differentiate into individual hematopoietic lineages. Orange; blood, purple; excretory, blue; muscle, green; mesoderm, pink; endothelial, AGM; aorta-gonad-mesonephros (Adapted from Evans et al., 2003).

The second wave of hematopoiesis is initiated by a cluster of cells that lines the dorsal vessel, known as the lymph gland (Wood and Jacinto, 2007) (Figure 1-12). The lymph gland is a specialised organ formed during embryogenesis and its progenitors share a common origin with the cells of the vascular (dorsal vessel) and excretory (pericardial nephrocytes) lineages, which all develop from cardiogenic mesoderm (Evans et al., 2003). The development of the cardiogenic mesoderm is reliant on signalling input by *dpp*, *fibroblast growth factor* (*FGF*), *Wingless* (*Wg*) and *Notch* (Frasch, 1995; Gisselbrecht et al., 1996; Wu et al., 1995). The hemocytes produced in the lymph gland are capable of giving rise to all three differentiated cell types that are released during the 3rd instar of larval development, however lymph gland hematopoiesis appears to be time-restricted as the organ disintegrates during metamorphosis (Shim, 2015). During this disintegration, the remaining lymph gland hemocytes are released into circulation. Interestingly, insulin/TOR signalling has been shown to play two important roles in the maintenance of blood cell progenitors and in

controlling the fate of prohemocytes (Benmimoun et al., 2012). In extreme environmental conditions or following immune challenge, larval tissue-resident macrophages can be supported by the newly differentiated hemocytes in the lymph gland (Gold and Brückner, 2014). The sudden release of hemocytes from the lymph gland during larval stages is due to the lymph gland bursting (Krzemien et al., 2007).

In adult *Drosophila*, the production of new blood cells has not been identified to date. It is thought hemocytes produced during the two waves of hematopoiesis persist into adulthood and throughout the flies lifetime (Holz et al., 2003).

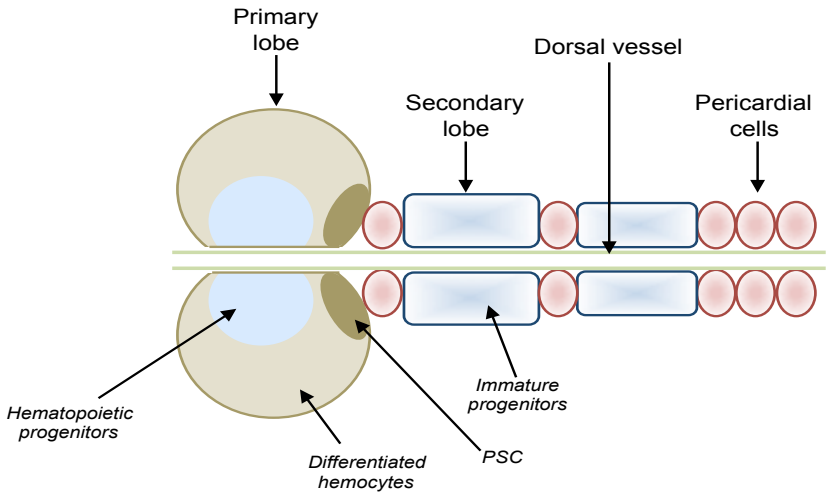


Figure 1-12: The lymph gland of *Drosophila* larvae
A schematic of the 3rd instar larval lymph gland. The lymph gland borders the dorsal vessel and pericardial cells separate each lobe. The primary lobe consists of three main regions; a cortical zone, where differentiated hemocytes are found, a medullary zone, where hematopoietic progenitors (prohemocytes lacking differentiation markers) are found and a PSC, which contains a specialised population of hemocytes. The secondary lobe usually consists of immature hemocytes. PSC; posterior signalling centre. (Adapted from Jung et al., 2005).

1.4.2. Transcriptional regulation of hematopoiesis

During hematopoiesis the differentiation of hemocytes subsets is reliant on lineage specific transcription factors (Figure 1-13). The *Drosophila* gene *serpent (srp),* a transcription factor of the GATA family is required for the formation of prohemocytes in the *Drosophila* embryo (Williams, 2007). During embryogenesis, early populations of prohemocytes in the head mesoderm express *srp*, which gives rise to two classes of blood cells, the plasmatocytes and crystal cells (Lebestky et al., 2000). *Srp* is also required later in development during hemocyte maturation. The receptor tyrosine kinase *PVR*, and its three ligands, *PVF1*, *2* and *3*, control the survival and migration of hemocytes in the tissues (Wood et al., 2006) as well as hemocyte proliferation in the lymph gland (Bond and Foley, 2009). Plasmatocyte differentiation requires two

transcription factors known as glial cells missing (*gcm*) and glial cell missing 2 (*gcm2*) (Alfonso and Jones, 2002). The differentiation of crystal cells is triggered by the expression of transcription factor, *lozenge* (*lz*) (Fossett et al., 2003). *lz* is also required in the embryo for the specification of crystal cells in the larval lymph gland (Wood and Jacinto, 2007) and the expression of *lz* in larvae also appears to be under the control of *Notch* signalling (Lebestky et al., 2003). The molecular control for the differentiation of lamellocytes remains widely unknown. However, it is thought that the transcription factor *collier,* also known as *knot* (*kn*), may be required in larvae development for the production of lamellocytes during parasitisation (Crozatier et al., 2004). The *Jak/STAT* pathway and *Toll* signalling pathways are also though to be implemented in lamellocyte proliferation and differentiation. The hyperactivation of *hopscotch*, the only Jak homolog, induces plasmatocyte proliferation, the differentiation of lamellocytes and the formation of melanised tumours (Agaisse and Perrimon, 2004). Disruption of Toll function within the lymph gland of *Drosophila* larvae can lead to hemocyte over-proliferation and differentiation defects (Qiu et al., 1998).

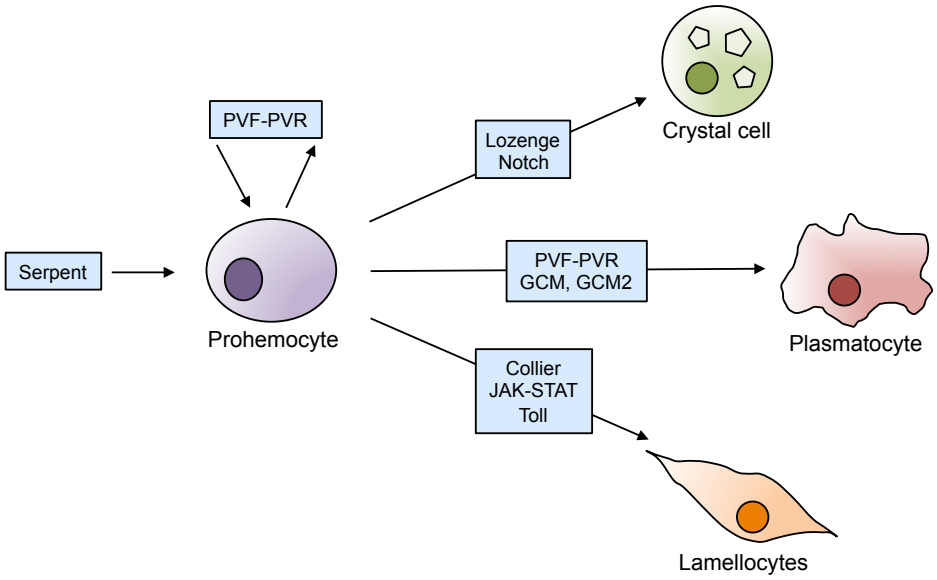


Figure 1-13: Regulation of hematopoiesis via transcription factors
Serpent is needed for hemocyte specification in the embryo. PVR and its ligands PVF1, 2 and 3, are important for hemocyte survival in the embryo and proliferation in the lymph gland. Differentiation of crystal cells requires the transcription factor *Lozenge*, which is under the control of Notch in *Drosophila* larvae. Plasmatocytes require the transcription factors GCM and GCM2. Lamellocytes need the transcription factor Collier, Jak/STAT and Toll signalling have also been implicated in lamellocyte differentiation and proliferation. PVF; platelet-derived/vascular endothelial growth factor, PVR; platelet-derived/vascular endothelial growth factor-receptor related, GCM; glial-cells missing, Jak; Janus kinase, STAT; signal transducers and activators of transcription (Adapted from Wood and Jacinto, 2007).

1.4.3. Hemocytes in *Drosophila* immune function

Drosophila hemocytes have two main cellular response mechanisms, phagocytosis and encapsulation.

Phagocytosis is carried out by the plasmatocytes and these cells are responsible for the removal of foreign intruders, apoptotic cells and cell debris (Williams, 2007). During *Drosophila* development, significant cell death occurs and hemocytes play an important role in the disposal of these apoptotic cells via the scavenger receptor *Croquemort* (*crq*) (Franc et al., 1999). The attachment of the phagocyte to the particle of interest is the first step of the phagocytosis process, followed by modifications to the cytoskeleton, internalisation and the destruction of the particle within phagosomes (Meister and Lagueux, 2003). Several receptor proteins have been shown to be involved in the process of phagocytosis including proteins related to *CD36* and *Eater*. *Eater*, an epidermal growth factor (EGF)-domain protein, is expressed exclusively on prohemocytes and plasmatocytes where it can bind and internalise a vast range of bacteria (Kocks et al., 2005).

Encapsulation is an important defence mechanism against invading parasites, which is mediated by lamellocytes in the larvae of *Drosophila* (Sorrentino et al., 2002). The encapsulation response is most often induced when the parasite is too big to be phagocytosed by plasmatocytes, for example following infection with the eggs of a parasitic wasp (Small et al., 2012). The invading parasite is detected by plasmatocytes that send signals to the lymph gland leading to increased proliferation and mass differentiation of lamellocytes from prohemocytes in the secondary lobes (Jung et al., 2005). The lamellocytes released from the lymph gland form a multi-layered case around the invading parasite, which becomes melanised by crystal cells and is eventually killed (Vlisidou and Wood, 2015).

Hemocytes have the ability to store defence molecules that can be released upon infection. For example, plasmatocytes can express immune molecules such as the blood clotting factor, *hemolectin*, or the Toll activating ligand *Spätzle* (Lemaitre and Hoffmann, 2007). Hemocytes are also able to play important signalling roles between immune tissues, produce cytokines, such as *upd3*, and AMPs following bacterial infection (Clark et al., 2011; Péan et al., 2017; Shia et al., 2009; Yang and Hultmark, 2016).

1.5. Epigenetic regulation and histone modifications

Since the early 1960s it has been known that histones, proteins that associate with DNA and package it into nucleosomes, are subject to post-transcriptional modification (Allfrey et al., 1964), such as methylation and ubiquitination, and play key roles in

epigenetic regulation. The core histones H2A, H2B, H3 and H4 join to form the nucleosome (Portela and Esteller, 2010). H1 is known as the linker histone, which does not form part of the nucleosome, but binds to the DNA linking two histone complexes and seals off the nucleosome where the DNA enters and leaves (Daujat et al., 2005). Epigenetic regulators can be divided into three main groups, based on their function: the epigenetic 'writers' place epigenetic marks onto DNA or histones and these marks can then be recognised by epigenetic 'readers' or removed by epigenetic 'erasers' (Falkenberg and Johnstone, 2014). Epigenetic marks are able to directly influence histone-DNA or histone-histone interactions and serve as a docking site for epigenetic readers (Bowman and Poirier, 2015). The binding of epigenetic reader to post-transcriptional modifications dictates the location and timing of recruitment of transcription factors and catalysis complexes (Andrews et al., 2016). Epigenetic mechanisms can modify gene expression and alter chromatin without changing the DNA sequence, and these epigenetic events are fundamental in maintaining normal cellular processes and development (Handy et al., 2011). Many human diseases, including cancer, have been implemented by the addition of the wrong type of epigenetic mark, or an epigenetic mark being added at the wrong time or in the wrong location (Sharma et al., 2010). Modifications to DNA or chromatin-associated proteins, such as histones, have important influences on chromatin structure and gene expression (Cedar and Bergman, 2009). These modifications can be grouped into two main categories: DNA modifications and histone modifications.

1.5.1. DNA modifications

DNA modifications have been best characterised by the methylation of cytosine at the 5-position of the cytosine ring, producing 5-methylcytosine (5mC). This deposition of the methyl group on cytosine is predominately carried out by a family of enzymes known as DNA methyltransferases (Tough et al., 2016) and is usually associated with gene repression. DNA methylation can regulate gene transcription by altering recognition sites of proteins that bind DNA or by disrupting transcription factor binding sites within gene promoters. Other newly identified DNA modifications include 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxycytosine (5caC) which can be formed following the oxidation of 5mC (Plongthongkum et al., 2014).

1.5.2. Histone modifications

Histones are subject to many types of modification, most occur on amino acids within the histone tails, which protrude from the nucleosome and provide an exposed surface for protein interactions (Jenuwein and Allis, 2001). These modifications play important roles in DNA repair and replication, transcriptional regulation and alternative

splicing (Portela and Esteller, 2010). Acetylation and methylation of histones are the most well studied modifications and many families of proteins are involved in adding, removing, or recognising these epigenetic marks, which are also known as writers, erasers and readers respectively. Acetylation of core histones occurs most frequently on multiple lysine residues, and is typically associated with gene expression. Histone acetylation is thought to alter the net charge of the histone and weaken its interaction with DNA, leading to the chromatin becoming more accessible to gene expression machinery and transcription factors (Hong et al., 1993). However, acetylated lysine residues can also act as recognition signals for reader proteins, predominantly to those containing acetyllysine recognition domains, to recruit proteins able to modify chromatin structure and gene transcription (Zeng and Zhou, 2002). The methylation of histones is more complex than that of acetylation as both lysine and arginine residues can be methylated (Alban et al., 2014) and the effects of methylation on gene transcription are far more varied. Interestingly, lysine residues can be mono-, di-, or tri-methylated (Martin and Zhang, 2005), whereas arginine can only be mono- or di-methylated (Bedford and Richard, 2005). Unlike acetylation, the process of methylation does not alter the charge of the histone, however it does recruit reader proteins to the DNA (Musselman et al., 2012). Methylation patterns can have diverse effects on transcriptional states, for example tri-methylation (Me3) of lysine (K) 4 in histone H3 (H3K4Me3) near the transcriptional start site is associated with gene expression, while H3K27Me3 further from the start site is usually associated with repression (Tough et al., 2016).

1.6. Bromodomain-containing proteins

Bromodomain-containing proteins (BCPs) can recognise acetyllysine residues on the tails of histones (Figure 1-14), specifically of histone H3 and H4, and regulate chromatin structure and gene expression (Fujisawa and Filippakopoulos, 2017). As BCPs recognise acetyllysine residues many of them are classed as epigenetic readers. The bromodomain (BD) family of proteins are comprised of a wide range of chromatin-associated proteins, including histone acetyltransferases (HATs) and chromatin-remodeling factors (Barbieri et al., 2013). BDs have been identified in a number of proteins with varying domain architecture and functions (Muller et al., 2011). The first BD was identified in the *Drosophila brahma* (*brm*) gene as a protein domain of approximately 110 amino acids (Haynes et al., 1992). In more recent years, these proteins have been divided into three main families, histone acetyltransferases, chromatin-remodeling complexes and bromodomain and extraterminal domain (BET) proteins (Josling et al., 2012).

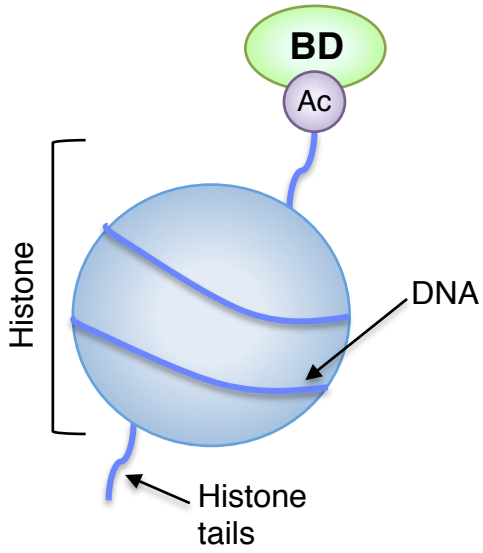


Figure 1-14: Bromodomain-containing protein (BCP) mechanism
The bromodomain (BD; green) of BCPs are able to recognise and bind acetyllysine resides (Ac; purple) on histone tails.

1.6.1. Histone acetyltransferases (HATs)

Histone acetyltransferases (HATs) are enzymes that acetylate conserved lysine amino acids in histones, they include *Gcn5* and *p300* cAMP-response-element binding (CREB)-binding protein associated factor (*PCAF*) in mammals. The bromodomain of the HAT complex is generally responsible for anchoring the HAT complex to acetylated chromatin, leading to acetylation of adjacent nucleosomes (Josling et al., 2012). Although some BCPs regulate gene expression only through their HAT activity, others can also regulate transcription directly through interactions with transcription factors (Bannister and Miska, 2000). In yeast, *Gcn5* contains both a HAT and a bromodomain that usually bind to acetylated histone H3, and to a lesser extent histone H4 (Sterner and Berger, 2000). The Gcn5 protein is a component of the SAGA (Spt-Ada-Gcn5 acetyltransferase) complex which is enriched on promoters of active genes and positively regulates transcription by RNA polymerase II (Robert et al., 2004). The mammalian p300 protein also has a bromodomain and a HAT domain, as well as other domains involved in protein binding. The bromodomain of *p300* binds to all core histones (preferentially H3), and is required for p300 to bind to chromatin. *p300* also has several other domains which allows them to bind a large number of proteins including TATA-binding proteins (TBP), RNA helicase A and RNA polymerase II, and this interaction allows them to act as transcriptional co-activators (Swope et al., 1996). It has also been shown that *p300* can regulate gene expression not just on acetylated histones, but also by recruiting RNA polymerase II and recognising non-histone proteins and non-histone substrates (Josling et al., 2012).

1.6.2. ATP-dependent chromatin-remodeling complexes

ATP-dependent chromatin-remodeling complexes utilise energy generated from ATP hydrolysis to locally alter and disrupt the association of histones with DNA, allowing nucleosome movement (Vignali et al., 2000). These complexes include *brm*, *SWItch/Sucrose Non-Fermentable* (*Swi2/Snf2*) and *Bramha-related gene 1* (*Brg1*). The bromodomain components within these complexes are critical for the recruitment of the complex to the genes that they regulate, and subsequent chromatin remodeling complexes can influence gene expression by mechanisms such as improving access to the promoter region for the transcriptional machinery (Hassan et al., 2001). The chromatin remodeling SWI/SNF complex regulates a subset of genes in yeast, *Drosophila* and mammals (Tang et al., 2010); it is found in the promoter region of these genes, and its chromatic remodeling activities allow for greater access for the transcriptional machinery (Peterson and Workman, 2000). The complex remains associated with RNA polymerase II during elongation and can play a role in transcriptional repression (Sudarsanam and Winston, 2000).This complex contains a catalytic ATP-dependent helicase component, known as *Swi2/Snf2*, which contains a bromodomain that preferentially binds to acetylated histone H3, particularly H3K14ac (Chatterjee et al., 2011). *Swi2/Snf2* show similarities to the human proteins BRG1 and the *Drosophila* protein brahma (Elfring et al., 1998). The transcriptional activator, *Brg1*, encodes a 205K nuclear protein and contains extensive homology to *Swi2* in humans (Khavari et al., 1993). Deletion of the bromodomain of *Swi2/Snf2* shows reduced binding to acetylated histones and as a result leads to a reduction in chromatin remodeling (Awad and Hassan, 2008).

1.6.3. Bromodomain and extraterminal (BET) proteins

The BET family is a novel class of transcription regulators (Loyola and Almouzni, 2004) including *Brd2*, *Brd3* and *Brd4* (De Riick et al., 2013). These proteins contain two bromodomains at their amino-terminal end as well as a conserved extraterminal (ET) domain at the carboxyl end that serves as a protein-protein interaction module (Josling et al., 2012). Similarly to other bromodomain proteins, BET proteins are primarily involved in regulating transcription through their interactions with a range of other proteins and retroviruses including murine leukemia virus (MLV). Members of the BET family have been recognised as essential genes in mediating inflammatory response, but also for the replication of viruses (Filippakopoulos et al., 2010). Interestingly, BET proteins are involved in gene regulation via a number of different mechanisms. *Brd2* (also known as RING3) is a mammalian BET protein with kinase activity that is associated with the promoters of a subset of cell cycle genes including

cyclins. *Brd2* binds as a dimer to acetylated residues in histone H4; its first bromodomain binds to H4K12ac and strongly to H1K74ac, and the second bromodomain binds as a dimer to H4K5ac and H4K8ac (Josling et al., 2012). *Brd2* functions as part of a complex, and much like many other bromodomain proteins, it is involved in the activation of cell cycle genes regulated by the cellular E2 factor (*E2F*) transcription factors and forms complexes with them. *Brd2* is also required to recruit the general transcription factor TATA binding protein (*TBP*) to the E2F complex (Peng et al., 2007). Mutations within both bromodomains in human embryonic kidney (HEK) 293 cells leads to an inhibition of transcription (LeRoy et al., 2008), suggesting *Brd2* plays a critical role in regulating transcription by facilitating the recruitment of transcription factors to target genes. A number of polymorphisms in *Brd2* have been linked to rheumatoid arthritis (Mahdi et al., 2009) and *Brd2* knockout mice show severe obesity and a reduction in an inflammatory response (Wang et al., 2009). *Brd4* (also known as MCAP) has a similar structure to *Brd2*, with an additional C-terminal motif that is involved in interacting with other proteins (Crowe et al., 2016). *Brd4* binds to acetylated histone H3 and H4, with the first bromodomain predominantly binding to acetylated H3 including H3K56ac but also H2AK85ac (Filippakopoulos et al., 2012), whereas the second bromodomain has a higher affinity for acetylated histone H4 (Vollmuth et al., 2009), but will bind strongly to various acetylated H2, H3 and H4. It is also able to bind to the active form of the positive transcription elongation factor b (PTEFb) complex (Schroder et al., 2011), an over-expression of *Brd4* causes PTEFb to increase phosphorylation of RNA polymerase II leading to increased transcription (Jang et al., 2005), showing *Brd4* is important for regulating transcription.

The human genome encodes 61 BCPs in 46 different proteins (Junwei and Vakoc, 2014) and some have now been implicated in disease processes (Sanchez and Zhou, 2009). *Brd4* plays an important role in various biological processes including functioning within the inflammatory response as a co-activator for the transcriptional activity of NF-κB and controlling viral gene transcription (Yang et al., 2005). *Brd2* has exhibited histone chaperone activity, in mice it is thought to be essential for embryonic development, and furthermore an association between *Brd2* and epilepsy in humans has also been described (Pal et al., 2003). *p300* has also been suggested to play a role in the *IL-6* signalling pathway, via an interaction between STAT3 amide-terminal domain and p300, stabilising the STAT3-p300/CREB-binding protein (CBP) complex (Hou et al., 2008). Recent studies have suggested BCPs may have functional roles in cancer cell survival and proliferation (Sanchez and Zhou, 2009). Research also demonstrated an important role for BET proteins in

modulating T-cell differentiation *in vitro*, therefore reducing the inflammatory response by transferred cells *in vivo* (Bandukwala et al., 2012).

1.6.4. Bromodomains as therapeutic targets

The development of novel pharmaceutical approaches that target inflammatory gene expression by interfering with the recognition of acetylated histones by BET proteins has become a main focus as a potential treatment for cancer and a number of inflammatory diseases including rheumatoid arthritis (Klein et al., 2016). A synthetic compound, known as I-BET has been shown to mimic acetylated histones and disrupt the chromatic complex responsible for the expression of inflammatory genes in activated macrophages (Nicodeme et al., 2010). The small molecule, JQ1 is a synthetic BET inhibitor and has been shown to suppress inflammatory responses in the brain of the 3xTg mouse model of Alzheimer's disease (Magistri et al., 2016). In the murine periodontitis model, systemic administration of JQ1 also significantly inhibits inflammatory cytokine expression (Meng et al., 2014).

1.7. Jumonji domain-containing proteins

Jumonji (Jmj) domain-containing proteins (JDCPs) are a family of proteins with functional roles in a number of biological processes including transcriptional repression and DNA/RNA repair through the demethylation of N-methylated nucleic acids (Figure 1-15) (Upadhyay et al., 2011). The jumonji family contains four distinct clusters: *JMJD2A-D* and *JARID1/2*, *JMJD3* and *UTX/UTY*, *JMJD1A-C* and *Hairless*, and finally *FBXL10/11* and *PHF2/8* (Cloos et al., 2008). The jumonji protein has a DNA binding domain (ARID), and two conserved jmj domains (jmjN and jmjC) (Takeuchi et al., 2006). In many species, there are known jumonji family proteins that have only the jmjC domain or both the jmjN and jmjC domains (Takeuchi et al., 2006). Several members of the jumonji family demethylate lysine residues in histones and are involved in chromatin regulation and/or transcription regulation (Klose et al., 2006). The function of the jmjN domain is widely unknown, however the jmjC domain is thought to play an essential role in histone demethylation and targets the removal of all three histone lysine methylation states (mono-, di- and trimethylation), most JmjC histone demethylase proteins characterised so far are capable of demethylating trimethylated lysines, and in many circumstances favour a trimethylated substrate (Tsukada et al., 2006). *Drosophila* Jmj shares high homology with the mammalian Jmj in the JmjN, JmjC and AT-rich interaction domains (Sasai et al., 2007). Data suggests Jmj in *Drosophila* play an important role in transcriptionally inactive chromatin and

controlling the expression of important developmental genes by modifying chromatin into silent transcriptional states (Sasai et al., 2007).

Jmj proteins, particularly JmjC-driven demethylase reactions have been identified in various biological processes, such as posterior development (Lan et al., 2007), prostate cancer (Xiang et al., 2007a), rRNA expression (Frescas et al., 2007), and androgen nuclear receptor-mediated gene expression (Yamane et al., 2006). It has also been shown as a transcriptional repressor and represses cyclin D1 transcription in the embryonic heart, which is required for normal cardiogenesis (Willems and Mercola, 2013), along with important roles in the development of multiple tissues. It has also been shown that H3K27 demethylation, specifically by JMJD3 catalytic activity, is critical for pro-inflammatory gene expression in human macrophages (Kruidenier et al., 2012).

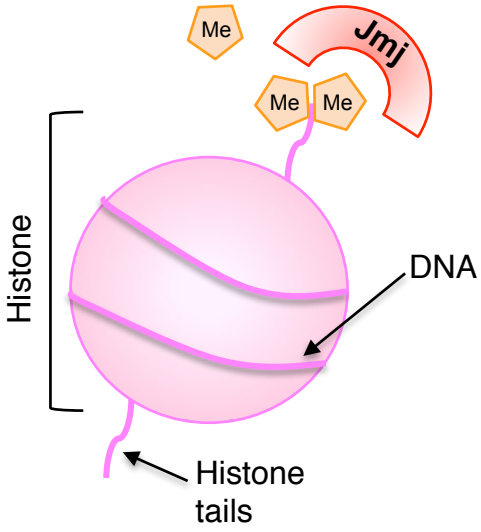


Figure 1-15: Jumonji domain-containing protein (JDCP) mechanism
The jumonji protein (Jmj; red) demethylates mono-, di- and tri-methylated residues (Me; orange) on histone tails.

1.8. Thesis outline

1.8.1. General hypothesis

female sterile (1) homeotic (*fs(1)h*), the sole bromodomain and extraterminal domain (BET) protein in *Drosophila*, plays an essential role in immunity and metabolism in the *Drosophila* fat body.

1.8.2. Thesis structure

Chapter 1 provided an overview of *Drosophila melanogaster*, the model organism used in this study, along with a detailed description of the *Drosophila* immune response and its regulation of metabolism in both fed and starved states. Chapter 1 also introduced two families involved in epigenetic regulation known as the bromodomain-containing proteins (BCPs) and jumonji domain-containing proteins (JDCPs). The materials and methods used throughout the course of this thesis are described in Chapter 2.

Chapter 3 investigates the roles of BCPs and JDCPs in *Drosophila* immune cells following bacterial infection. This includes the use of tissue-specific RNAi knockdowns of BCPs and JDCPs and infecting the flies with *Listeria monocytogenes* or *Francisella novicida*, one Gram-positive and one Gram-negative intracellular bacteria. This chapter describes the survival screens that were carried out using two fat body drivers (*c564-Gal4* and *r4-Gal4*) and two hemocyte drivers (*Hml$^{\Delta}$Gal4, UAS-2xeGFP* and *crq-Gal4*). The survival screens led to the identification of *female sterile (1) homeotic, fs(1)h*, being short-lived following fat body knockdown. *fs(1)h* is a bromodomain-containing protein, and the survival screens suggested it is essential in the fat body for *Drosophila* survival.

Chapter 4 further describes the immune role of *fs(1)h* when it is knocked down in the *Drosophila* fat body, following on from the data obtained in Chapter 3. This chapter also investigates the novel role *fs(1)h* has in *Drosophila* immunity, metabolism, insulin signalling and nutrient sensing. Much of the data obtained in this chapter suggests many of the phenotypes observed are *foxo*-dependent and that *fs(1)h* in the fat body has a key *in vivo* function in promoting and regulating systemic insulin signalling activity.

The data obtained from Chapter 4 shows that knocking down *fs(1)h* in the *Drosophila* fat body leads to a range of phenotypes, mainly due to FOXO hyperactivation. Chapter 5 aims to investigate the role of FOXO in the flies with *fs(1)h* knocked down in the fat body. The data in this chapter shows that removing one copy of *foxo* in flies lacking *fs(1)h* in the fat body can rescue many of the phenotypes

observed, confirming much of it is *foxo*-dependent. However, data in Chapter 5 also shows some of the phenotypes, particularly following bacterial infection, can not be rescued by *foxo* heterozygosity, suggesting there is also a *foxo*-independent role for *fs(1)h* in the fat body. Furthermore, Chapter 5 begins to investigate potential physical or transcriptional interactions between fs(1)h, FOXO and AKT.

Chapter 6 investigates the effects of the pharmacological inhibitor, I-BET151, and siRNAs for mammalian BET proteins in the human cell line, THP-1, on insulin signalling. The first part of this chapter uses the small-molecule inhibitor, I-BET151, to treat THP-1 cells and investigate any changes in the insulin-signalling pathway by measuring protein levels of activated AKT and FOXO3a. The second part of this chapter uses small interfering RNAs (siRNAs) to individually knockdown three of the mammalian bromodomain and extraterminal domain (BET) proteins known as *bromodomain-containing protein 2*, *3* and *4* (*Brd2, 3, 4*), in THP-1 cells. This part includes checking the efficiency of each siRNA knockdown and measuring activated AKT and FOXO3a protein levels.

Chapter 7 includes a detailed discussion of the data shown in this thesis in relation to the known literature in both *Drosophila* and mammals, as well as an outlook on future work.

Chapter 2

Materials and Methods

2.1. Maintenance of *Drosophila melanogaster*

2.1.1. Fly stock maintenance

Drosophila stocks and experimental flies were kept in incubators at 18°C, 25°C, or 29°C, with 60% humidity. They were housed with a running 12hour light: 12hour dark cycle to help maintain the flies' natural circadian rhythm. Individual fly stocks that were not being used experimentally, were kept in duplicate in vials containing approximately 8ml of fly food in the 18°C incubator until required, at which point they were expanded and placed at 25°C. Fly stocks were checked at regular intervals to ensure stocks were healthy and of the correct phenotypic characterisations. Stocks were also transferred to fresh food vials every 3-4 weeks without CO_2 anaesthesia.

2.1.2. Fly food preparation

Flies were kept on food containing 10% weight to volume Brewer's yeast, 2% polenta and 0.8% agar (MP Biomedicals), 8% fructose (Sainsbury's supermarkets Ltd), 0.5% nipagin (Methyl 4-hydroxybenzoate, Sigma-Aldrich) in 15% ethanol, and 0.75% propionic acid (Sigma-Aldrich).

Agar and polenta was added to three-quarters of the total water, brought to the boil and simmered for 2 minutes whilst being stirred constantly to prevent burning. The heat was then reduced before adding yeast and fructose, along with the remaining water allowing the food to cool down. Once the temperature had cooled to below 70°C, the nipagin (in 15% ethanol) and propionic acid were added as antimicrobial and antifungal agents. Approximately 8ml of food was pumped into each vial, and sealed immediately with a cotton bung to prevent any contamination. Following cooking, the sealed vials were kept at room temperature or 4°C until required, if kept at 4°C the vials were left to warm up at room temperature before placing flies on it.

2.1.3. Identifying males and females

Adult *Drosophila* were anaesthetised under a light microscope on a porous CO_2 gas pad, moving and separation of flies was carried out using a soft bristled paintbrush to prevent damaging the flies. The identification of males and females was distinguished by a number of physical characteristics:

(i) Females tend to be larger than males.

(ii) Males show darker abdominal pigmentation and genitalia than females.

(iii) Male flies have sex combs, which are located on the first pair of legs and used to attach to the female during mating. The sex combs can be identified under a light microscope as thick black hairs.

2.1.4. Virgin collection

Fly vials containing hatching pupae were cleared of any adults and left for new flies to eclose. Virgin females were collected up to 6 hours post eclosion, as during this period they will not have mated. Virgin female can be visually identified through their pale colouring and a dark spot in the abdomen, known as the meconium, which consists of waste products remaining from pupation. Collected virgin females were kept in separate vials at 25°C for three days before carrying out any crosses, to ensure the collected females were virgins. Any vials seen to contain eggs or larvae and consequently not virgins, were discarded.

2.2. *Drosophila* Stocks

During this thesis a number of *Drosophila melanogaster* lines were used to carry out experiments that are described below.

2.2.1. Wild type stocks

The w^{1118}, white eyed, wild type fly line (with isogenic X, second and third chromosomes), was the predominant control fly line used. These flies carry an insertion in the *white* allele leading to white-eye colour. The w^{1118} flies were also used when crossing experimental flies to generate heterozygote controls.

2.2.2. The *Gal4*-UAS system

The *Gal4*- upstream activating sequence (UAS) system, developed by Brand and Perrimon in 1993, is commonly used in *Drosophila* to achieve spatially and temporally restricted gene expression (McGuire et al., 2004). In this system, a *P* element carrying the transcriptional activator in yeast, *Gal4*, leads to the expression under the control of endogenous tissue-specific enhancers that allows tissue-specific expression of *Gal4*. *Gal4* can recognise and bind UAS sequences in the DNA and facilitate transcription of the target transgene that has been cloned downstream of the UAS leading to tissue-specific expression or knockdown (Figure 2-1).

The *Gal4*-UAS system was used throughout this thesis, most frequently for tissue-specific knockdown of target genes in the *Drosophila* fat body and hemocytes. Table 2-1 shows the genotypes of the transgenic fly stocks used in this thesis.

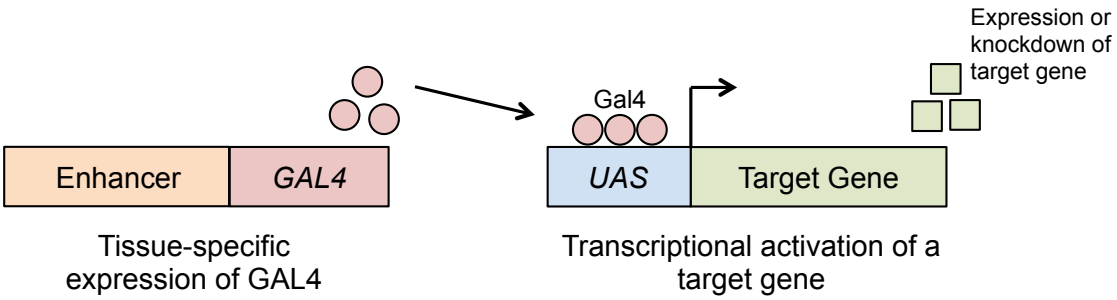


Figure 2-1: The *Gal4*-UAS system
The yeast transcription factor, *Gal4*, activates transcription of its target gene by binding to the UAS. The driver line provides tissue-specific *Gal4* expression (left) and the gene of interest is under UAS control allowing expression or knockdown of the gene of interest (right). UAS; upstream activating sequence.

2.2.3. Temperature sensitive *Gal80* (*Gal80^{ts}*) system

Temperature sensitive *Gal4*-repressor *Gal80* (*Gal80^{ts}*) uses alterations in temperature to regulate the *Gal4*-UAS system. Ubiquitously expressed *Gal80^{ts}* in *Drosophila* binds to the carboxy-terminal of *Gal4* and represses transcriptional activation at 18°C. However, when shifted to 29°C the *Gal80^{ts}* is degraded and the *Gal4* can activate the expression or knockdown of the specific transgene downstream (Figure 2-2).

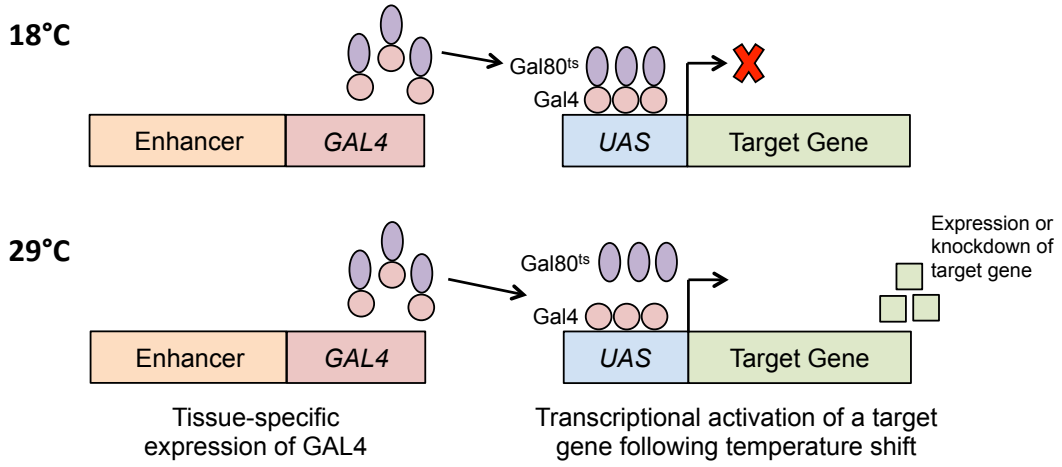


Figure 2-2: Temperature sensitive *Gal80* in the *Gal4*-UAS system
At 18°C, *Gal80* binds to the *Gal4* repressing transcriptional activation (top). At 29°C, *Gal80* is released allowing *Gal4* to activate transcription (bottom).

2.2.4. RNA interference (RNAi)

To investigate the roles of specific genes in detail, RNAi lines of various genes of interest were obtained. The RNAi lines used in this thesis are shown in Table 2-2. The RNAi lines were linked to upstream activating sequences (UAS) and when used in combination with a *Gal4* driver tissue-specific knockdown of the target gene is achieved. The *Gal4*-UAS system drives the expression of a hairpin RNA (hpRNA),

which is in turn cut by *Dicer*, an enzyme that produces small interfering RNAs (siRNAs). The siRNAs bind to mRNA and lead to sequence-specific degradation of the target mRNA, which results in the loss of translation of the gene of interest in a specific tissue of interest (Figure 2-3).

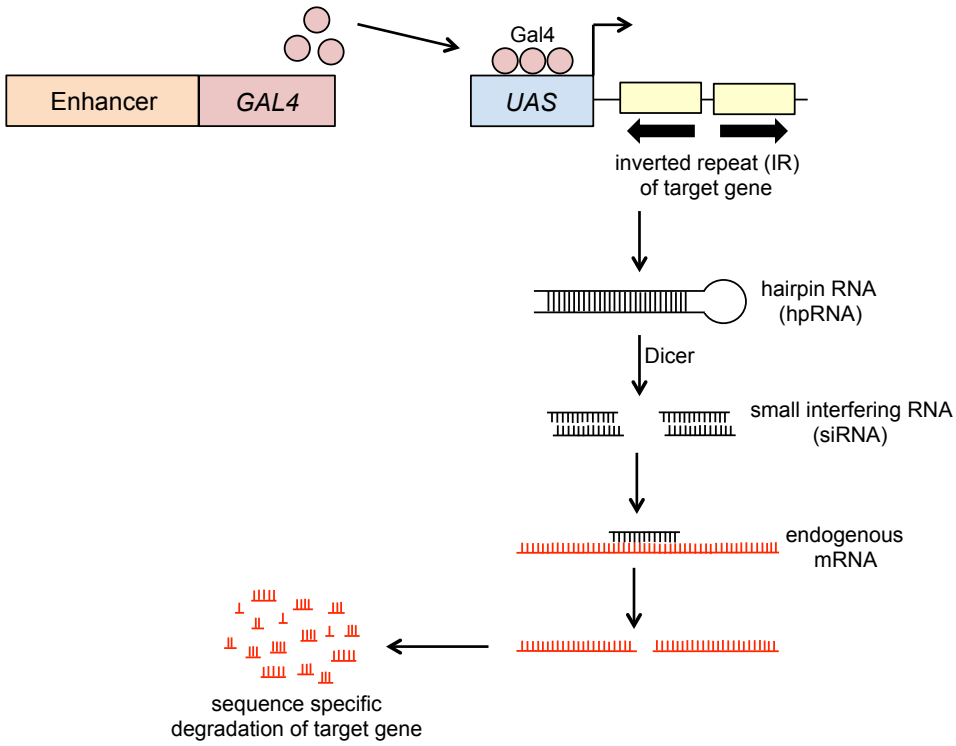


Figure 2-3: RNA interference (RNAi) in *Drosophila* using *Gal4*-UAS
The *Gal4*-UAS system is used to drive the expression of a hairpin RNA (hpRNA), the double-stranded RNAs are processed by the enzyme Dicer into small interfering RNAs (siRNAs) which sequence specifically degrades the mRNA of the target gene. This degradation leads to a loss of translation of the target gene ubiquitously or in a specific cell type such as hemocytes or the fat body (Adapted from VRDC, 2017).

2.2.5. Balancer Chromosomes

Balancer chromosomes are an essential tool when working with *Drosophila*; they are used to prevent recombination, maintain deleterious mutations in stocks and allow the tracking of chromosomes following *Drosophila* crosses (Rubin and Lewis, 2000). A number of fly lines used in the thesis have the addition of balancer chromosomes in order to prevent lethal or sterile mutations being lost from a *Drosophila* population (Muller, 1918) and stop multiple alleles on the same chromosome from being separated by meiotic recombination (Miller et al., 2016). Balancer lines used in this thesis are shown in Table 2-3.

2.2.6. Experimental flies and crosses

Flies required for experiments were crossed and kept at 25°C, whereas stocks were maintained at 18°C. Unless stated otherwise, male flies were used for all experiments.

Fly crosses were carried out in standard vials, containing standard fly food. Approximately 5 virgin females of the required tissue-specific *Gal4* driver line and 4 males of the w^{1118} line as the driver line control or the necessary UAS responder line was crossed for each experiment carried out. *Gal4* crosses were maintained at 25°C, with progeny being ready to collect approximately 10 days following the setup of the cross. An example of the control and experimental crosses are shown in Figure 2-4.

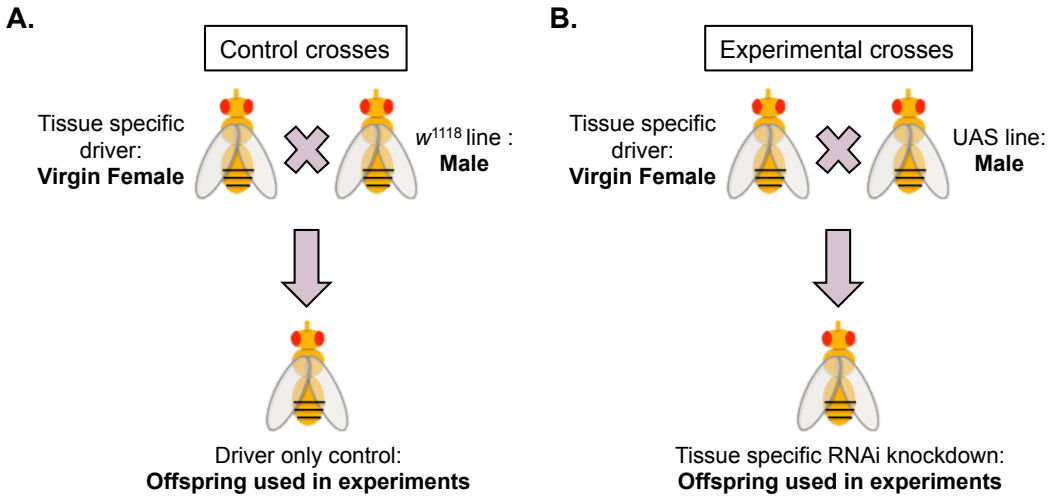


Figure 2-4: An example of the *Drosophila* crosses set in this thesis
A. For each experiment a control cross was set up, virgin females of each tissue-specific *Gal4* driver were crossed to w^{1118} male flies. The male offspring from these crosses were aged for 5-7 days and used for the required experiments. **B.** The experimental crosses were set up along side the control crosses, but this time the virgin females of each tissue-specific *Gal4* driver were crossed to each of the UAS-RNAi lines or other required lines. The male offspring from the crosses with the required genotype were aged for 5-7 days and used for the required experiments.

2.3. *Drosophila* stocks used in this thesis

The transgenic fly lines used in this thesis are listed in Table 2-1 below.

Fly Stock	Chromosomal Location	Purpose
w^{1118};*c564-Gal4*	II	Fat body expressed *Gal4* line.
w^{1118};;*r4-Gal4*/Tm6c Sb^{1}	III	Fat body expressed *Gal4* line.
w^{1118};Hml^{Δ}*Gal4*, UAS-2xeGFP	II	Hemocyte/plasmatocytes expressed *Gal4* line. Can also be used to visualised hemocytes (2x eGFP).
w^{1118};;*crq-Gal4*/Tm6c Sb^{1}	III	Hemocyte/plasmatocytes expressed *Gal4* line.

Table 2-1: List of transgenic *Gal4* driver lines

The UAS-RNAi lines used in this thesis are listed in Table 2-2 below.

Stock Centre	Stock Number	Knockdown Line (CG number)	Gene Name	Chromosome Location
VDRC	22108	UAS-*CG5383*-IR	*Phosphatidyl-serine receptor (PSR)*	III
VDRC	22689	UAS-*CG5383*-IR	*Phosphatidyl-serine receptor (PSR)*	III
VDRC	24740	UAS-*CG32346*-IR	*Enhancer of bithorax (E(bx))*	III
VDRC	33446	UAS-*CG1966*-IR	*ATP-dependent chromatin assembly factor large subunit (ACF)*	I
VDRC	33447	UAS-*CG1966*-IR	*ATP-dependent chromatin assembly factor large subunit (ACF)*	III
VDRC	35962	UAS-*CG8569*-IR	-	II
VDRC	37671	UAS-*CG7229*-IR	*testis-specifically expressed bromodomain-containing protein-2 (tbrd-2)*	III

VDRC	37720	UAS-*CG5942*-IR	*brahma (brm)*	III
VDRC	37721	UAS-*CG5942*-IR	*brahma (brm)*	III
VDRC	46645	UAS-*CG32346*-IR	*Enhancer of bithorax (E(bx))*	I
VDRC	100383	UAS-*CG42799*-IR	*dikar*	II
VDRC	100735	UAS-*CG10897*-IR	*toutatis (tou)*	II
VDRC	101311	UAS-*CG1845*-IR	*Bromodomain-containing protein, 140kD (Br140)*	II
VDRC	101737	UAS-*CG5206*-IR	*bonus (bon)*	II
VDRC	102664	UAS-*CG13597*-IR	*testis-specifically expressed bromodomain-containing protein-1 (tbrd-1)*	II
VDRC	102885	UAS-*CG15319*-IR	*nejire (nej)*	II
VDRC	103830	UAS-*CG9088*-IR	*little imaginal discs (lid)*	II
VDRC	104121	UAS-*CG5653*-IR	-	II
VDRC	104879	UAS-*CG14514*-IR	*Bromodomain-containing 8 (Brd8)*	II
VDRC	105115	UAS-*CG15319*-IR	*nejire (nej)*	II
VDRC	105986	UAS-*CG5640*-IR	*Utx histone demethylase (Utx)*	II
VDRC	106119	UAS-*CG17603*-IR	*TBP-associated factor 1 (TAF-1)*	II
VDRC	107312	UAS-*CG42799*-IR	*dikar*	II
VDRC	107321	UAS-*CG1815*-IR	-	II
VDRC	107378	UAS-*CG30417*-IR	*testis-specifically expressed bromodomain-containing protein-3 (tbrd-3)*	II
VDRC	107819	UAS-*CG2982*-IR	-	II
VDRC	107868	UAS-*CG15835*-IR	*Lysine (K)-specific demethylase 4A*	II

			(Kdm4A)	
VDRC	107992	UAS-*CG7154*-IR	-	II
VDRC	108618	UAS-*CG11375*-IR	*polybromo*	II
VDRC	108662	UAS-*CG2252*-IR	*female sterile (1) homeotic (fs(1)h)*	II
VDRC	108848	UAS-*CG10133*-IR	-	II
VDRC	108943	UAS-*CG4107*-IR	*Gcn5 acetyltransferase (Gcn5)*	II
VDRC	109290	UAS-*CG3654*-IR	*Jumonji, AT rich interactive domain 2 (Jarid2)*	II
VDRC	109294	UAS-*CG43320*-IR	-	II
VDRC	109295	UAS-*CG11033*-IR	*Lysine (K)-specific demethylase 2 (Kdm2)*	II
VDRC	109417	UAS-*CG7200*-IR	-	II
VDRC	110158	UAS-*CG33182*-IR	*Lysine (K)-specific demethylase 4B (Kdm4B)*	II
VDRC	110485	UAS-*CG7460*-IR	-	II
VDRC	110808	UAS-*CG31132*-IR	*BRWD3*	II
VDRC	51227	UAS-*CG2252*-IR	*female sterile (1) homeotic (fs(1)h)*	III

Table 2-2: RNAi lines used in this thesis

Balancer lines used in this thesis are shown in Table 2-3 below.

Balancer	Chromosomal Location	Marker
w^{1118} *Fm7a*	X	Bar eyes
w^{1118}; If/Sm6a	II	Bar eye (If), Curly (Sm6a)
w^{1118};; TM2/Tm6c Sb^{1}	III	Ebony (TM2) Stubble (Tm6c Sb^{1})
w^{1118}; If/Sm6a; TM2/Tm6c Sb^{1}	II and III	Bar eye, Curly, ebony and Stubble

Table 2-3: Balancer lines used in this thesis

Other lines used in this thesis are shown below in Table 2-4.

Fly Line	Chromosomal Location	Purpose
w^{1118}; $FOXO^{GFP}$	II	GFP-tagged FOXO fusion protein
w^{1118};; $foxo^{\Delta 94}$/Tm6c Sb^{1}	III	*foxo* mutant flies

Table 2-4: Other fly lines used in this thesis

2.4. Life span assays

There were a number of different types of life span assays used in this thesis. The life span of the flies was measured under normal physiological conditions, following infection and following starvation.

2.4.1. Physiological Survival

Male flies were collected following eclosion for 3-4 days to obtain between 20-30 age-matched flies per genotype and placed onto new fly food. All survival experiments were carried out at room temperature, 25°C or 29°C.

In all survival assays carried out, each vial was checked daily for any fly deaths that may have taken place and numbers were recorded. Food vials were also kept horizontally during the assay to prevent the flies getting stuck in the food.

2.4.2. Starvation survival

For starvation survivals, male flies were collected from the relevant crosses and maintained for 5-7 days on standard fly food at 25°C. Following this time, groups of approximately 20 flies per genotype were transferred onto vials of new, standard fly food as a control or vials of agar (with 1% PBS), allowing the flies to have access to water and prevent desiccation. These experiments were usually carried out at room

temperature or 25°C. Each vial was checked every two hours during the day for any fly deaths that had taken place and numbers were recorded.

2.4.3. Infection survival

Male flies were collected following eclosion for 3-4 days to obtain enough flies per genotype and then aged for between 5-7 days at 25°C. For all infection survivals, flies were put into groups of 20-30 individuals, one group was an uninjected control, and the other groups of flies were injected into the abdomen with sterile PBS as a wounding control, or infected with *Listeria monocytogenes* (*L. monocytogenes*) or *Francisella novicida* (*F. novicida*). All infection survival experiments were carried out at 25°C or 29°C.

2.5. Injection assays

2.5.1. Calibration for injection

Fly injections were carried out with microinjection needles that were produced from borosilicate glass capillaries (Kwik-Fil) and a needle puller (Model PC-10, Narishige). Injections were performed using a PicospritzerR III, and the injection volume was calibrated by expelling a drop of liquid from the needle into a pot of mineral oil and halocarbon oil (both Sigma). The expelled drop was measured using the microscope graticule to obtain a final injection volume of 40 nanolitres (nl).

2.5.2. Bacterial culture and infection

The bacterial stocks of *L. monocytogenes* and *F. novicida* (U112 strain) were stored at -80°C. Small quantities of these stocks were removed using a plastic scraper and dipped into the selected media, *L. monocytogenes* was placed in Tryptic Soy Broth (TSB, Sigma Aldrich) and *F. novicida* in Brain, Heart Infusion (BHI, Oxoid) media. The individual cultures were grown over night at 37°C, either still (*L. monocytogenes*) or shaking (*F. novicida).* The following day, the cultures were centrifuged at 12,000g for 10 minutes and the supernatant was discarded. The bacterial pellets were re-suspended in sterile PBS (Corning) and the optimal density (OD) was measured at the wavelength of 600nm (OD600), the bacterial stocks were diluted to an OD of 0.1 prior to injections. Once the needle was calibrated (see 2.5.1) flies were anaesthetised on CO_2 and injected into their abdomen with 40nl of liquid.

Following bacterial infection, flies were placed back into vials at 29°C, counted daily for survival assays or six hours post infection the flies were homogenized in 100µl TRIzol (TRI reagent, Sigma) and stored at -20°C ready for the generation of cDNA and RT-qPCR analysis. The flies were homogenized six hours post infection in

order to observe maximum AMP induction. For some experiments, infected flies (and controls) were also kept at 29°C for 24 hours and then homogenized in 75µl 2x Laemmli loading buffer (100mM Tris [pH6.8], 20% glycerol, 4% SDS, 0.2M DTT). The samples were stored at -80°C until required for Western blot analysis.

2.5.3. Insulin injection

Male flies of the appropriate genotypes were collected and aged for 5-7 days at 25°C. Flies were starved for 1 hour prior to injections, and using the microinjection needles the flies were injected into the abdomen with 40nl of PBS or human insulin (Sigma) resuspended in PBS at a low dose (1.28µg/ml) or a high dose (6.4µg/ml). The flies were homogenised 10 minutes post-injection in 75µl 2x Laemmli loading buffer. The samples were stored at -80°C until required for Western blot analysis.

2.5.4. 2-NBDG injection

Male flies of the appropriate genotypes were collected and aged for 5-7 days at 25°C. Using the microinjection needles, the flies were injected into the abdomen with 40nl of PBS or 2-NBDG (50mM, Cayman chemical) resuspended in sterile PBS and placed at 29°C for 1 hour prior to imaging. Images were acquired using a Leica SP5 microscope. Images were obtained using the Leica LAS-AF software with either the 10x numerical aperture (NA) 0.4 objective or the 20x Dry NA 0.5 objective. All images had the resolution of 1024 x 1024 pixels, at a scan speed of 400Hz. There was a 4 line average carried out during imaging to obtain the highest quality images. Tile scanning was used in order to image whole flies, as it was not possible to image a whole fly in a single tile. Following imaging, the Leica LAS software automatically merged the two image tiles, producing one image of a whole fly.

2.6. Fat body dissection

Male flies of the appropriate genotypes were aged for 5-7 days prior to fat body dissection. Flies were anaesthetised on CO_2, then one at a time moved onto the dissecting plate and secured by placing a fine dissecting pin (Austerlitz Insect Pins, Size 1) through the thorax. The legs and wings were removed using dissection scissors, and the abdomen was secured by placing a second dissecting pin through the genital segment. The fly was covered in chilled sterile PBS, and any bubbles were removed using a plastic pipette. Two pairs of forceps were used to open the abdomen along the ventral midline, and the guts and gonads were removed. The cuticle connecting the thorax and abdomen was cut and any heart or tracheal tissue still attached to the abdomen section was removed and discarded. The abdominal cuticular surface, which is internally covered in a layer of fat body, was taken for RT-

qPCR analysis. The dissected fat body samples were stored with 3 dissected flies in 100µl TRIzol (Sigma) for RT-qPCR analysis. All samples were stored at -20°C until required. This dissection technique was adapted from Krupp and Levine (2010).

2.7. RT-qPCR (Real Time-quantitative Polymerase Chain Reaction)

2.7.1. Generation of cDNA from whole flies and dissected fat body samples

RNA extractions were carried out using TRIzol (Sigma), three flies per sample were homogenised in 100µl of TRIzol and left to sit at room temperature for 5 minutes. Chloroform (20µl, VWR) was added to each sample for washing. Samples were mixed thoroughly and left at room temperature for 3 minutes, the samples were then centrifuged at 12,000rpm for 15 minutes at 4°C. The upper aqueous phase was removed from each of the microfuge tubes and placed into new tubes; the lower, organic phase was discarded. Once each sample had been transferred into new tubes, 50µl isopropanol (VWR) was added and mixed well in order to precipitate the RNA. The tubes were left for 10 minutes at room temperature, and then spun at 4°C for 10 minutes at 12,000rpm. The supernatant was removed and the RNA pellets were washed in 200µl of 75% ethanol (VWR). The samples were centrifuged for 5 minutes at 13,000rpm and the ethanol was removed and discarded. The RNA pellets were re-suspended in 40µl DNAse1 mix (Table 2-5).

DNAse 1 Components	Quantity per sample	Manufacturer
RNAse-free water	34µl	Ambion
10x DNA buffer (Buf. DNAse1 + $MgCl_{2)}$	4µl	Thermo Scientific
DNAse1, RNAse-free	2µl	Thermo Scientific

Table 2-5: DNAse1 Mix Components

Each RNA sample was then incubated for 30 minutes at 37°C to remove any residual genomic DNA. Following incubation, 4µl 25mM EDTA (Thermo Scientific) was added to each sample to bind magnesium ions and samples were incubated at 68°C for 10 minutes. Reverse transcription was carried out by incubating 10µl of the DNAse-treated RNA with 1µl Random Hexamers (Thermo Scientific) at 68°C for 5 minutes and then incubated on ice for 5 minutes. Following the 5-minute incubation, 9µl of the reverse transcription mix (Table 2-6) was added to each sample. Samples were left to incubate at room temperature for 10 minutes and then for 1 hour at 37°C. Finally, the samples were heated at 68°C for 10 minutes to inactivate the enzymes. The 20µl of cDNA generated was stored at -20°C until use in RT-qPCR experiments.

Reverse Transcription Components	Quantity per sample	Manufacturer
M-MuLV buffer (Buf RT)	4µl	Thermo Scientific
10mM dNTPs (deoxyribonucleotides)	2µl	Thermo Scientific
RNAse inhibitor (Ribolock)	1µl	Thermo Scientific
Reverse Transcriptase (RevertAid)	1µl	Thermo Scientific
RNAse-free water	1µl	Ambion

Table 2-6: Reverse Transcription Mix Components

2.7.2. RT-qPCR

Following the production of the cDNA (20µl), half of each sample was taken from each tube and combined to make the first of eight standards in a 1:3 dilution series. The remaining 10µl of cDNA was mixed in a 1:40 dilution with 1x TE buffer (10mM Tris, 1mM EDTA). The RT-qPCR was run using 4.8µl diluted cDNA along with 5µl 2x Sensimix SYBR green no-ROX (Bioline) and 0.2µl of a 10µM primer pair mix of choice, with a total reaction volume of 10µl. The primer stocks were stored at a concentration of 100mM in TE buffer at -20°C, and further diluted to 10µM working stocks containing both left and right primer pairs (Table 2-7).

A Corbett Rotor-Gene 6000 was used to run the RT-qPCR, and the cycle settings used were: Hold 95°C for 15 minutes, then 40 cycles of 95°C for 15 seconds, 57°C for 30 seconds, 72°C for 30 seconds, and finally a melting curve from 72°C to 95°C.

The primer sets used in this thesis are shown below in Table 2-7.

Primer	Left	Right
α-tubulin	GATTCCGGTGACGGTGAG	GATGCACACCTATCCGCTCT
Rpl1	TCCACCTTGAAGAAGGGCTA	TTGCGGATCTCCTCAGACTT
Gal4	GCAGCATTCTGGAACAAAGA	GGACAATTGGATCTCCCAAG
fs(1)h	GAAAAAGCAGAAACTGGAGAA	TGCTCGAAGCGGACTCAT
AttA	CACAATGTGGTGGGTCAGG	GGCACCATGACCACCATT

CecA1	TCTTCGTTTTCGTCGCTCTC	CTTGTTGAGCGATTCCCAGT
Def	TTCTCGTGGCTATCGCTTTT	GGAGAGTAGGTCGCATGTGG
Dipt	ACCGCAGTACCCACTCAATC	CCCAAGTGCTGTCCATATCC
Dro	CCATCGAGGATCACCTGACT	CTTTAGGCGGGCAGAATG
Drs	GTACTTGTTCGCCCTCTTCG	CTTGCACACACGACGACAG
Mtk	TCTTGGAGCGATTTTTCTGG	TCTGCCAGCACTGATGTAGC
Hsl	CTTGGAAATACTTGAGGGGTTG	AGATTTGATGCAGTTCTTTGAGC
bmm	GTCTCCTCTGCGATTTGCCAT	CTGAAGGGACCCAGGGAGTA
plin1	GCGTTCTATGGTAGCCTTCAG	GCGTCCGGATAGAAAGCTG
plin2	GCAGAATGGCAAGAGTTCTGA	ACTGTGTGTAGGACTGGATCCTC
InR	GCACCATTATAACCGGAACC	TTAATTCATCCATGACGTGAGC
DILP2	ATCCCGTGATTCCAAGAGAA	GCGGTTCCGATATCGAGTTA
DILP3	CCGAAACTCTCTCCAAGCTC	TGAGCATCTGAACCGAACT
DILP5	GCCTTGATGGACATGCTGA	AGCTATCCAAATCCGCCA
DILP6	CCCTTGGCGATGTATTTCC	CACAAATCGGTTACGTTCTGC
ImpL2	GCCGATACCTTCGTGTATCC	TTTCCGTCGTCAATCCAATAG
foxo	ACTTTCCCGTGGGCAATC	CGCCGTAGCTCATCTCCT
Pepck	GGATAAGGTGGACGTGAAG	ACCTCCTGCGACCAGAACT
Thor	CAGGAAGGTTGTCATCTCGGA	GGAGTGGTGGAGTAGAGGGTT
dpp	CCTTGGAGCCTCTGTCGAT	TGCACTCTGATCTGGGATTTT
eiger	CGACGAGTTCCAAAAGGAGT	GTCGTCGTCCTCCTCATC
upd1	GCACACTGATTTCGATACGG	GCACACTGATTTCGATACGG
upd2	CGGAACATCACGATGAGCGAAT	TCGGCAGGAACTTGTACTCG
upd3	ACTGGGAGAACACCTGCAAT	GCCCGTTTGGTTCTGTAGAT

CCHa2	TGCTACTGGTCGTTATCTGCAC	TAGGCCTGGCATCCCTTT
Gbp1	CAGCACAGAACAGATCACCAG	GCAGCACAGTGGTGTTTTCA
Gbp2	AAACGGCGGACATGGATA	TGGAAAACGACCGAAGACA

Table 2-7: RT-qPCR primer sequences used in this thesis

2.8. Western blot

Samples of three flies per microcentrifuge tube were homogenised in 75µl of 2x Laemmli loading buffer (100mM Tris [pH 6.8], 20% glycerol, 4% SDS, 0.2 M DTT) and the samples were stored at -80°C until required. Samples were left to defrost at room temperature, then along with the protein ladder (Blue Protein Standard, Broad Range; New England Biolabs) heated to 85°C for 5 minutes and vortexed. The protein ladder and sample lysate was loaded at 5µl per lane in a 4-12% polyacrylamide gel (Invitrogen, Life Technologies). The gel was run using 1x MES SDS Running buffer (20x: 50mM MES, 50mM Tris base, 0.1% SDS, 1mM EDTA) at room temperature for 35 minutes at 300mAmps, 200 volts and 50 watts. The protein from the gel was transferred onto nitrocellulose (Amersham Protran, GE Healthcare) or polyvinylidene difluoride (PVDF; Immobilon Transfer Membrane, Millipore) membrane. The membrane and filter paper (Chromatography paper, Whatman) was cut to 8cm x 7cm and soaked in transfer buffer (25mM Tris, 192mM glycine, 20% (v/v) methanol) along with the sponges to keep the gel, membranes and filter paper held in place. The PVDF membrane was soaked in 100% methanol (VWR) prior to being soaked in transfer buffer. The methanol hydrates the hydrophobic PVDF membrane and improves transfer and protein binding. Once soaked in transfer buffer, the transfer equipment was assembled, the red plastic was placed on the bottom, as the negatively-charged proteins run towards the positive charge, then the sponge, followed by the filter paper, membrane, gel, a second piece of filter paper, a second sponge and finally the top, black plastic. The gels were then placed into the transfer cell, containing ice cold transfer buffer and the transfer was ran out at 4°C for 1 hour 20 minutes, at 300 mAmps, 400 volts and 50 watts.

Once the transfer was complete, the membranes were removed from the transfer equipment and placed in a container of blocking solution of milk (Non fat dry milk, Cell Signalling) and put on a rocker at room temperature for 1 hour. The membranes were washed 5 times, each for 3 minutes using TBS-T (Tris buffered saline, 0.1% Tween20) and incubated with the required primary antibody. The primary antibodies were added at 1:1,000 in bovine serum albumin (BSA; Albumin Fraction V, AppliChem), and the membranes were left at 4°C on the rocker overnight. Following

membrane washing with TBS-T (5 times, 3 minutes each), the secondary antibodies were added at 1:5,000 in milk and the membranes were placed on the rocker at room temperature for 2 hours. Membranes were then washed again in TBS-T (5 times, 5 minutes each) and the proteins were detected with either Supersignal West Pico- or Femto- Chemiluminiscent Substrate (both Thermo Scientific) depending on the primary antibody used. A BioRad Molecular Imager was used to expose the images, and the band densities were analysed using BioRad Image Lab software.

The primary antibodies used for *Drosophila* work in this thesis were as followed:

Antibody	Manufacturer	Concentration Used
Anti-S505-phosphorylated *Drosophila* Akt	Cell Signalling, #4054	1:1,000
Anti-p70 S6 Kinase	Cell Signalling, #9206	1:1,000
Anti-GFP D5.1 XP	Cell Signalling, #2956	1:1,000
Anti-Relish-C	Developmental Studies Hybridoma Bank, 21F3	1:1,000
Ubiquitin (P4D1)	Cell Signalling, #3936	1:1,000
Anti-*α-tubulin*	Developmental Studies Hybridoma Bank, 12G10	1:10,000

Table 2-8: Primary antibodies used for *Drosophila* Western blots

The secondary antibodies used for *Drosophila* work in this thesis were as followed:

Antibody	Manufacturer	Concentration Used
HRP anti-rabbit IgG	Cell Signalling, #7074	1;5,000
HRP anti-mouse IgG	Cell Signalling, #7076	1:5,000

Table 2-9: Secondary antibodies used for *Drosophila* Western blots

2.9. Coomassie staining

Samples of three flies were homogenised in 75µl of 2x Laemmli loading buffer and ran on a 4-12% polyacrylamide gel, as described above in section 2.8. Once the gel had run, it was fixed in Fixing Solution (Table 2-10) for 30 minutes on the rocker at room temperature. The gel was then stained in Coomassie Working Solution (Table 2-11) for 45 minutes on the rocker at room temperature. After 45 minutes, the gel was

destained using Destaining Solution (Table 2-10) for 2-3 hours on the rocker and then imaged using an Epson Scanner.

	Fixing Solution (100ml)	Destaining Solution (100ml)	Manufacturer
Methanol	50ml	45ml	VWR
Acetic Acid	10ml	10ml	VWR
H_2O	40ml	45ml	

Table 2-10: Fixing and destaining solution quantities for Coomassie

Coomassie Working Solution	Manufacturer	Quantity
Methanol	VWR	250ml
Coomassie stain (concentrated)	See Table 2-12	15ml
Acetic Acid	VWR	50ml
H_2O		200ml

Table 2-11: Coomassie working solution quantities

Coomassie Concentrated Stain	Manufacturer	Quantity
Brilliant Blue R-250 Dye (BBR)	Thermo Scientific	12g
Methanol	VWR	300ml
Acetic Acid	VWR	60ml

Table 2-12: Coomassie concentrated stain quantities

2.10. Thin layer chromatography (TLC)

Thin layer chromatography (TLC) was used to quantify stored triglyceride levels in whole flies. Groups of 10 flies, in quadruplicate per genotype were anaesthetised using CO_2 and placed in 100µl of 3:1 ratio of chloroform:methanol (both VWR). The fly samples were centrifuged at 13,000rpm at 4°C for 3 minutes and then homogenised. In order to generate a standard curve and quantify the whole fly triglycerides, a set of standards were prepared using lard (Sainsbury's supermarkets Ltd) dissolved in 3:1 chloroform:methanol. Standards (2µl) and samples (20µl) were then loaded onto a silica gel (Merck) as show in Figure 2-5. A 4:1 ratio of hexane: ethyl ether (both VWR) was used for the mobile phase of the TLC. Gels were then stained using the general oxidising stain, ceric ammonium heptamolybdate (CAM; cerium (V) sulphate hydrate (Sigma-Aldrich), ammonium molybolate tetrahydrate (Honeywell) and Sulphuric acid (H_2SO_4, VWR), left to dry and baked at 80°C for approximately 20 minutes. Once the

gel had baked, it was imaged using an Epson Scanner and triglyceride levels were analysed using the ImageJ software.

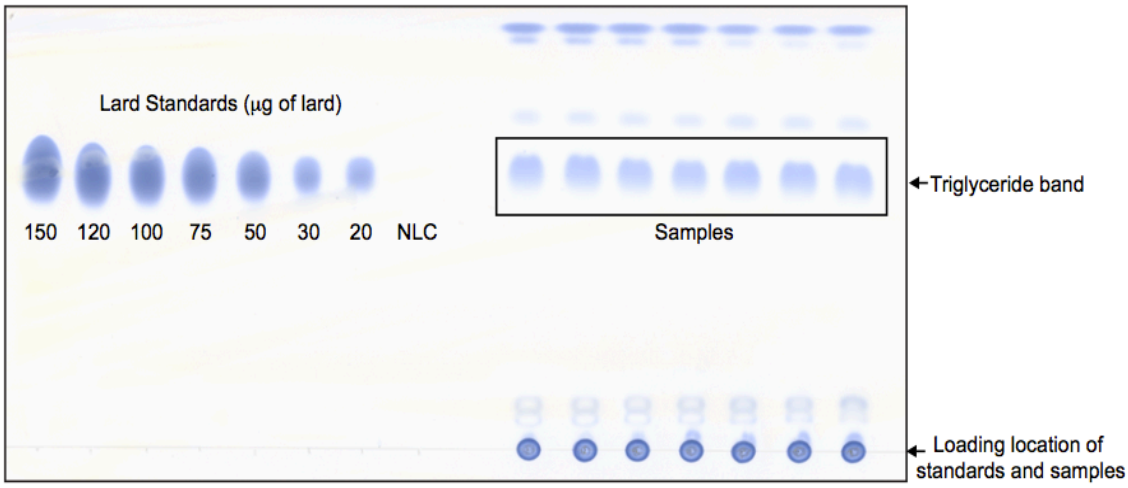


Figure 2-5: TLC plate layout
A TLC silica gel. All standards and samples were loaded at the bottom of the gel (as indicated by the arrow). The lard standards are shown on the left hand side with the micrograms (µg) of lard, the box on the right shows the triglycerides present in the samples. The standards and triglyceride bands were quantified using ImageJ.NLC; no lard control.

2.11. LipidTOX staining

Whole flies of the required genotypes were fixed for 1 hour in 4% paraformaldehyde (PFA, Sigma) and 0.1% Triton X-100 (Sigma), the flies were then pulled apart and fixed for a further 30 minutes in 4% PFA and 0.1% Triton X-100. Samples were then washed 3 times with 1x PBS (Corning) and 0.1% Triton X-100. During the last wash, DAPI (Life Technologies, 1:1,000) was added and incubated for 10 minutes at room temperature. The samples were washed 3 times with 1x PBS, and incubated with HCS LipidTOX Deep Red (Life Technologies, 1:200) in 1x PBS for 2 hours at room temperature in the dark. Finally, the samples were mounted on glass cover slips in Fluoromount-G (eBioSciences). Images were acquired using a Leica SP5 microscope. Images were obtained using the Leica LAS-AF software using the 10x numerical aperture (NA) 0.4 objective and all images had the resolution of 1024 x 1024 pixels, at a scan speed of 400Hz. There was a 4 line average carried out during the imaging to obtain the highest quality images.

2.12. Glucose, trehalose and glycogen quantification

Circulating sugars (glucose and trehalose) and stored carbohydrate (glycogen) levels in *Drosophila* were quantified using a colorimetric assay with a glucose reagent containing glucose oxidase and peroxidase. The amount of glucose in each sample was assumed to be proportional to the development of a red compound detected after oxidisation of the glucose by glucose oxidase in the presence of peroxidase. Flies

were starved for 1 hour and then homogenised in 75µl TE + 0.1% Triton X-100 in groups of three flies per genotype, at least 5 samples per genotype were prepared and samples were frozen at -80°C until required. When required the samples were removed from the -80°C, left to thaw and then heated for 20 minutes at 90°C to inactivate the fly enzyme, trehalase. Samples were loaded at 5µl into flat bottom 96-well tissue culture plate (Greiner bio-one).

Each fly sample was run four times, firstly alongside water for the calculation of fly background absorbance, secondly with glucose reagent (Sentinel diagnostics), thirdly with glucose reagent plus trehalase (from porcine kidney, Sigma, 1:100) to allow for the measurement of both glucose and the glucose released from trehalose. Finally, glucose plus glycogen levels were measured by adding glucose reagent plus amyloglucosidase (Sigma Aldrich, 1:100). Samples were treated with trehalase or amyloglucosidase in order to take into account any trehalose or glycogen present along with glucose in whole flies. In order to analyse the sugar levels, glucose standards were also prepared (1:2 dilution, 10-series dilution) and loaded along with the samples. Each lane was also run with H_2O in order to control for spectrophotometer detection differences. The 96-well plate layout is shown in Figure 2-6. Plates were incubated at 37°C for 10 minutes before reading with a Wallac spectrophotometer at a wavelength of 490nm.

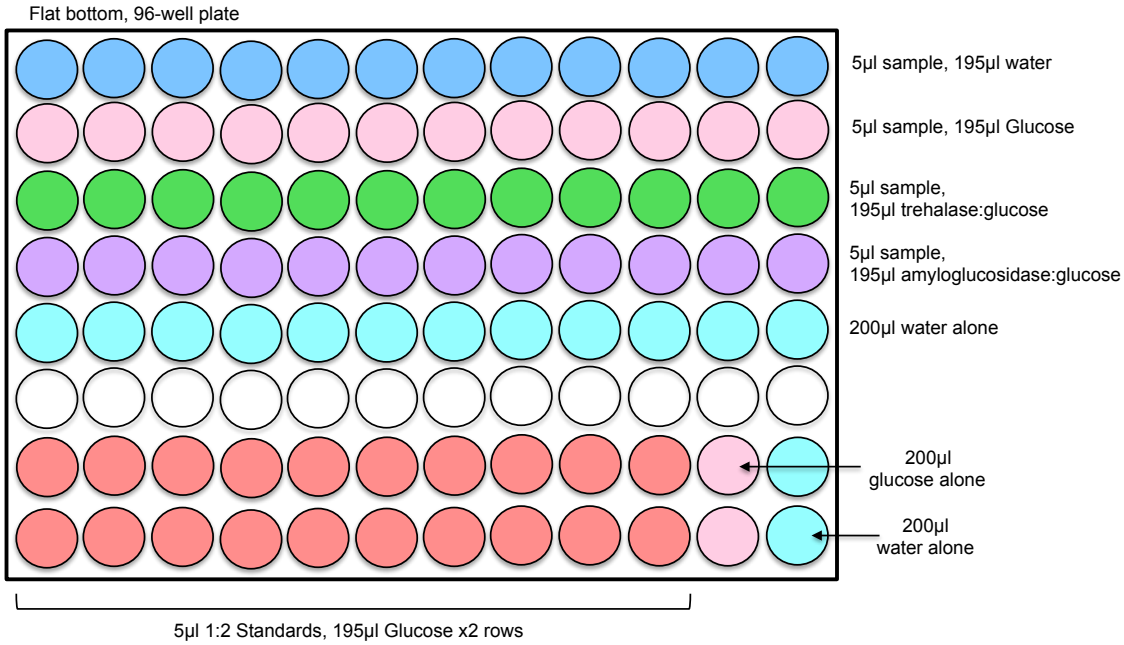


Figure 2-6: 96-well plate layout for trehalose, glucose and glycogen analysis
To measure circulating sugars and stored carbohydrate levels, 96-well plates were set up as shown. See text for details.

2.13. Genomic DNA preparation

Following the collection of flies, three flies per sample were homogenised in 150µl Buffer A (100mM Tris-HCl, 100mM EDTA, 100mM NaCl, 0.5% SDS). Each sample was incubated at 65°C for 30 minutes. Following incubation, 300µl LiCl/KAc (6M LiCl, 5M KAc) was added and the samples were placed on ice for 10 minutes. Samples were centrifuged at 12,000rpm at room temperature for 15 minutes. The supernatant was moved into new microcentrifuge tubes and 240µl of isopropanol was added, mixed and samples were centrifuged at 12,000rpm at room temperature for 15 minutes. The supernatant was discarded; the pellets were washed with 200µl 70% ethanol and then removed, the pellets left to dry and resuspended in 150µl TE. The samples were stored at -20°C until required for PCR.

2.13.1. Genomic DNA PCR

Following the generation of genomic DNA, 1µl of each sample was taken and mixed with 2µl Standard Taq buffer (New England Biolabs), 0.4µl 10mM dNTPs (New England Biolabs), 2µl 16S ribosomal DNA primers (Sigma, Table 2-13), 0.4µl Taq (New England Biolabs) and 14.7µl distilled H_2O. The PCR was run on the following programme: 94°C for 10 minutes, 35 cycles at 94°C for 1 minute, 54°C for 1 minute and 72°C for 2 minutes, and finally 72°C for 5 minutes. The samples were run on a 1% gel with 3µl of SYBR Safe DNA Dye (Life Technologies) at 110 volts for 35 minutes and DNA bands were visualised using a Safe Imager Transilluminator gel documentation system (Thermo Scientific).

Primer	Left	Right
16S	AGAGTTTGATCCTGGCTCAG	GGTTACCTTGTTACGACTT

Table 2-13: 16S ribosomal DNA PCR primers

2.14. Smurf assay

Flies were maintained on standard fly food until the day of the Smurf assay. Standard fly food was dyed using Blue dye no.1 (SPS Alfachem) at a concentration of 2.5% (wt/vol). Flies were maintained at 29°C on the dyed food for a 2-hour time period starting from 9am. A fly was classed as a 'Smurf' when the dye coloration could be observed outside of the digestive tract. Flies with a blue digestive tract were classed as 'eaten' and flies with no internalised blue colouration were classed as 'not eaten'. A minimum of 20 flies was used per genotype, per experiment.

2.15. *In vitro* experiments

2.15.1. Cell culture

The human monocytic cell line, THP-1 (BioCat, 140554) was maintained in complete Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies), supplemented with 2mM Glutamine (Life Technologies), 10mM Hepes (Life Technologies), 10% Fetal Bovine Serum (FBS, Life Technologies) and 1% penicillin/streptomycin (Life Technologies) at 37°C in 5% CO_2, at a concentration range of 0.5-1 x 10^6 cells per ml. The cell line was sub-cultured every three days.

2.15.2. Treating THP-1 cells with I-BET151

The THP-1 cells were seeded in 6-well plates at a density of 0.5 x 10^6 per 2ml of RPMI media (Life Technologies) supplemented as shown above. Unstimulated cells were treated with 2µl of 1µM GSK1210151A (I-BET151) compound (in DMSO) or with DMSO alone (as the control), with a final DMSO concentration of 0.1%. Following 24-hour treatment with the inhibitor or DMSO alone, the THP-1 cells were harvested by moving the cell media from the appropriate wells into microcentrifuge tubes and spun for 1 minute at 13,000rpm. The supernatant was removed and the pellet was re-suspended in 100µl Radioimmunoprecipitation assay (RIPA) lysis and extraction buffer (ThermoFisher Scientific). Samples were then frozen at -80 ^oC until required for Western blot analysis.

2.15.3. Western blot of samples from the THP-1 cell line samples

Cells were harvested and placed into 100µl LDS Sample Buffer and NuPage Reducing Agent. Samples were heated to 85^oC for 5 minutes, vortexed briefly and 5µl of cell samples and 5µl SeeBlue Plus2 Pre-stained ladder (Thermo Scientific) were loaded into 4-12% polyacrylamide gels (Invitrogen, Life Technologies) and run in MES buffer (NuPage MES SDS Running Buffer) for 40 minutes at 200 volts. Following electrophoresis, the transfer was prepared with the following set-up of the transfer: blotting pads (x3), filter paper (Invitrogen), gel, nitrocellulose transfer membrane (Invitrogen, Pore Size 0.45µl), filter paper, blotting pads (x3). Blotting pads, membranes and filter paper were all soaked in 1x transfer buffer (Invitrogen Transfer Buffer, 20x) prior to use. The transfer was run out at room temperature, for 1 hour at 30 volts. Membranes were blocked in 3% Bovine Serum Albumin (BSA; Sigma) on the rocker for 2 hours at room temperature. The primary antibodies (Table 2-14) were added to BSA and left on the rocker overnight at 4^oC. The membranes were washed 3 times, 10 minutes each with TBS-T and the secondary antibodies (Table 2-15) were added in Odyssey blocking buffer (Li-Cor, 927-40000), wrapped in tin foil to protect

from the light, and placed on the rocker at room temperature for 1 hour. The membranes were washed in TBS-T 3 times for 10 minutes each time. Excess TBS-T was removed and the membrane was laid face down on the imager. The membranes were imaged using the Odyssey imaging system (Li-Cor) to identify the bands.

The primary antibodies used for the THP-1 cell line work were as followed:

Antibody	Manufacturer	Concentration Used
Phospho-AKT (Ser473)	Cell Signalling, #9271	1:1,000
FoxO3a (D19A7)	Cell Signalling, #12829	1:1,000
β-Actin	Sigma Aldrich, A5316	1:10,000
BRD2	Bethyl, A302-583A-M	1:1,000
BRD3	Bethyl, A302-368A	1:1,000
BRD4	Cell Signalling, #13440	1:1,000

Table 2-14: Primary antibodies used for THP-1 cell line Western blots

The secondary antibodies used for the THP-1 cell line work were as followed:

Antibody	Manufacturer	Concentration Used
Alexa fluor 680 Donkey Anti Rabbit	Life Technologies, A10043	1:5,000
Alexa fluor 680 Rabbit Anti Mouse	Life Technologies, A21065	1:5,000

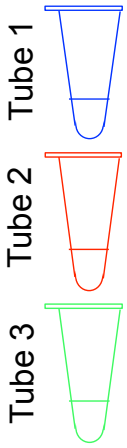
Table 2-15: Secondary antibodies used for THP-1 cell line Western blots

2.15.4. Small interfering RNA (siRNA)

Small interfering RNA (siRNA), an RNAi tool, was used to induce short term silencing of bromodomain-containing proteins 2, 3 and 4 (*Brd2*, *3*, *4*) in the THP-1 human cell line. THP-1 cells were placed into 6-well plates at 0.5 x 10^6/ml in RPMI media and left overnight. The cells were removed from each well and placed into 15ml falcon tubes and centrifuged at 400rcf for 4 minutes at room temperature and resuspended in 1.8ml RMPI media per falcon tube.

2.15.4.1. siRNA Preparation

Before transfecting the THP-1 cells, the siRNA reagents were prepared as shown in Figure 2-7. Viromer GREEN (Lipocalyx, VG-01BL-00), the transfection reagent used, binds small oligonucleotides to efficiently knockdown the gene of interest in a number of cell lines, including THP-1 cells.



- 10µM BRD2, 3 or 4 siRNA solution from 20µM BRD2, 3 or 4 stock
- buffer GREEN (1:2 dilution)

- 10µM non-targeting siRNA from the 20µM non-targeting siRNA stock
- buffer GREEN (1:2 dilution)

- 12µl Viromer GREEN pipetted onto the wall of a sterile RNAse free tube
- 1080µl buffer GREEN

Note: vortex for 3-5 seconds

Figure 2-7: siRNA tube preparation
To start the siRNA protocol, Tube 1 contained the required siRNA (Brd2, 3 or 4), Tube 2 contained the non-targeting siRNA, which was used as the control treatment and Tube 3 contained Viromer, the polymer-based transfection reagent (Lipocalyx, 2017).

The reagents used for siRNA preparation are shown below in Table 2-16.

siRNA	Stock Information
BRD2	ON-TARGETplus SMARTPool, Dharmacon, Human BRD2, cat# L-004935-00
BRD3	ON-TARGETplus SMARTPool, Dharmacon, Human BRD3, cat# L-004936-00
BRD4	ON-TARGETplus SMARTPool, Dharmacon, Human BRD4, cat# L-004937-00
Non-targeting siRNA	ON-TARGETplus Non-targeting Pool, Dharmacon, #D-001810-10-05

Table 2-16: **siRNA stock information**

2.15.4.2. Transfection

The transfection step was carried out in triplicate in RNAse/DNAse free, sterile tubes (Figure 2-8). Each complexation was incubated at room temperature for 15 minutes and 200µl from each complexation tube was added to the respective wells for a final volume of 2ml per well. The cells were incubated for 3 days at 37^{o}C with 5% CO_2.

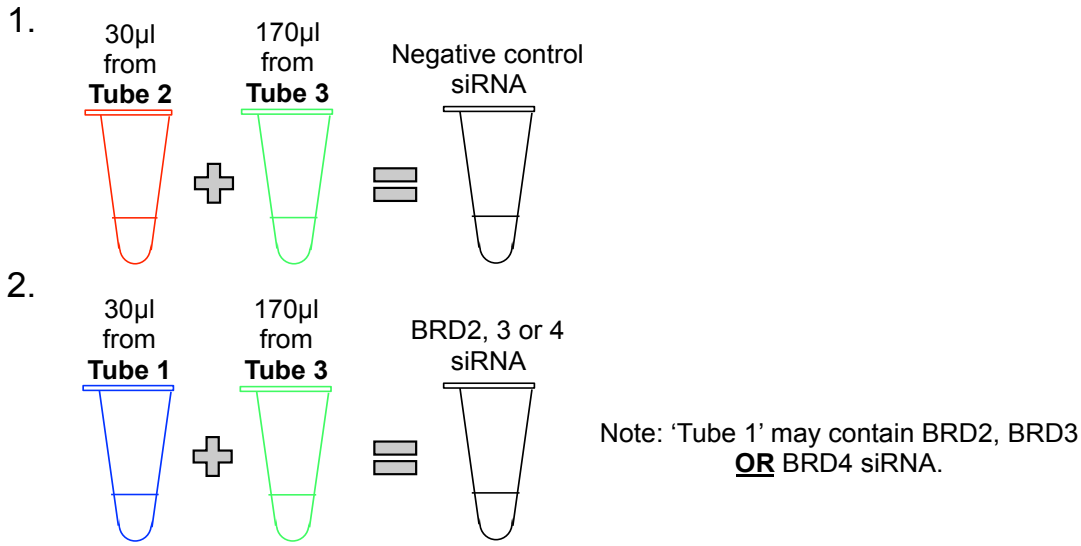


Figure 2-8: siRNA transfection set up
The tubes from Figure 2-6 above were used to set up the correct siRNAs to treat the THP-1 cells. Top: Complexation (1) indicates the non-targeting siRNA, as the negative control. Bottom: Complexation (2) indicates the siRNA to knockdown the required gene.

2.15.4.3. Cell harvest and Western blot

Following the three-day incubation time with the siRNA, the THP-1 cells were taken from each well and put into 15ml Falcon tubes, each well was washed with 1ml sterile PBS, and the wash-out was added to each respective Falcon tube. Each tube was centrifuged at 400rcf for 4 minutes and the cell pellet was resuspended in 1ml sterile PBS (Gibco) and transferred into 1.5ml microcentrifuge tubes. The tubes were centrifuged at 1,500rcf for 2.5 minutes, the PBS was removed and the cell pellets were resuspended in 40µl RIPA buffer. The cell lysates were incubated on ice for 30 minutes, with vortexing taking place every 10 minutes; the supernatants were transferred to new centrifuge tubes and stored at -80^{o}C. Western blots were carried out as described above in 2.15.3.

The primary antibodies and secondary antibodies used for the human cell line Western blots are shown above in Table 2-14 and Table 2-15, respectively.

2.16. Statistical analysis

2.16.1. Survival analysis

All survival experiments included approximately 20-30 flies per genotype. From the initial infection survival screen, percentage survival and median survival for each genotype was calculated and graphs were plotted in GraphPad Prism (GraphPad Software). For any of the phenotypes that showed large variation (two days or more) away from the control genotypes the survival experiment was repeated. Lifespan experiments and starvation survival experiments were repeated four to six times.

2.16.2. RT-qPCR analysis

The gene expression concentrations were obtained using a standard curve that was generated for each primer set used (Figure 2-9A) and a melt curve was used to indicate the presence of cDNA in each sample (Figure 2-9B). The expression levels of individual samples were normalised to the expression level of the housekeeping gene *α-tubulin.* RT-qPCR data was analysed when the R^2 value for standard linearity was 0.99 or above, and if each primer had an efficiency of 0.75 or above. Each experiment included at least four samples, with three flies per sample, and individual experiments were repeated three times. Data analysis using unpaired t-tests or 2-way ANOVAs were carried out using GraphPad Prism to determine statistical significance.

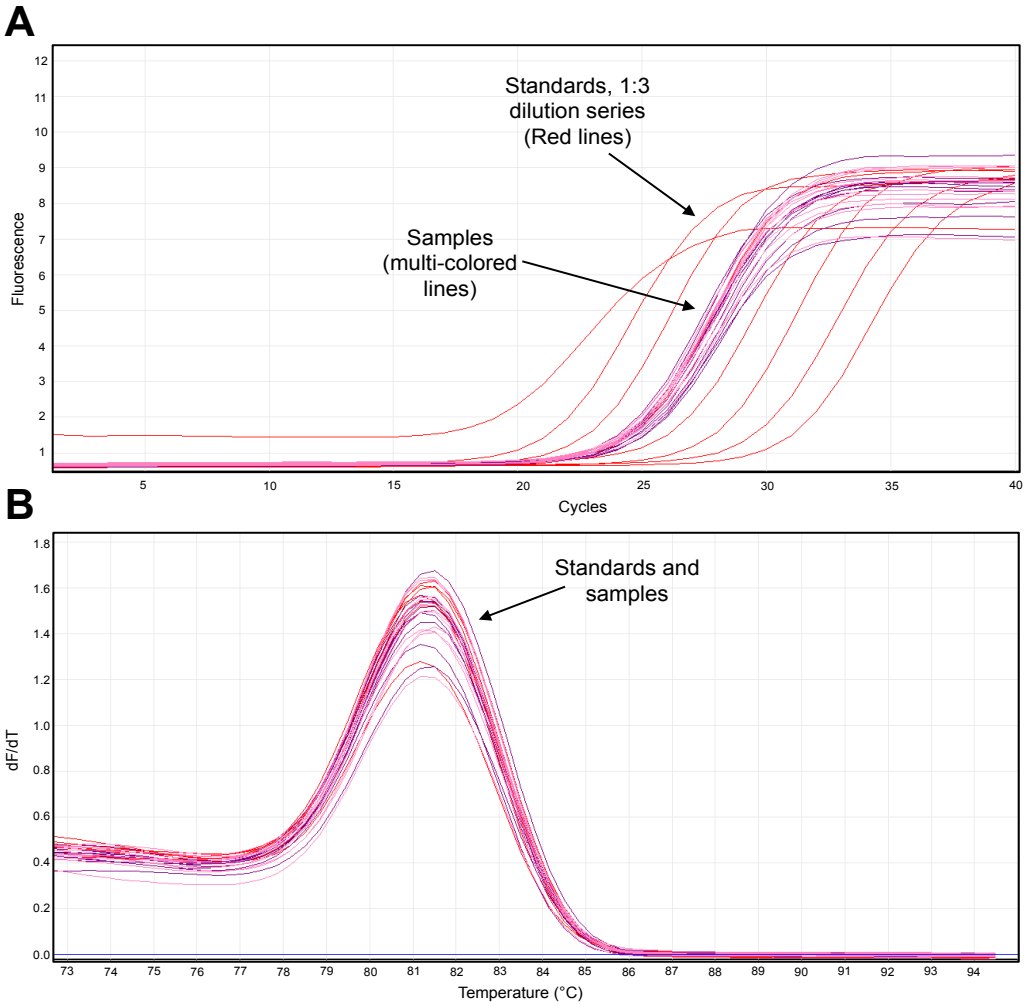


Figure 2-9: Standard curve and melt curve for RT-qPCR
An example of a cDNA standard curve and melt curve. **A.** The standards are indicated by the red lines and the multi-coloured lines indicate the samples. **B.** The melt curve shows the presence of cDNA in each of the standards and samples. dF/dT: derivative of fluorescence vs. derivative temperature, which shows the changes in fluorescence caused by temperature changes leading to double stranded DNA melting to single stranded DNA.

2.16.3. Western blot analysis

For all Western blots, 3 flies were taken per sample. Following Western blot, the image was opened in ImageJ and the rectangle tool was used to draw a box around each band of interest to obtain the density of each band. The background was removed and ImageJ calculated the relative area and density. The same process was carried out to obtain the density of the loading control bands (*α-tubulin* or actin) and the amount of protein was calculated relative to the loading control. Each experiment was repeated at least three times, graphs were generated and data was analysed using unpaired t-tests in GraphPad Prism.

2.16.4. Thin Layer Chromatography (TLC) gel analysis

A total of 10 flies were included in each sample, in quadruplicate for each of the TLCs, each TLC was also repeated at least three times to confirm findings. Using the set of standards that were prepared using lard, a standard curve was generated from the known concentrations of lard in order to calculate the approximate amount of lard (in µl) in each fly sample. In samples where no lard could be detected, graphs were annotated with non-detected (n.d). The TLC analysis was carried out using Bio-Rad Image Lab software and an unpaired t-test was preformed to identify statistical significance in GraphPad Prism.

2.16.5. Glucose, trehalose and glycogen colorimetric assay analysis

Glucose, trehalose and glycogen data was exported from the spectrophotometer in excel files showing the absorption at 490nm. Unpaired t-tests were used to identify statistical significance. A standard curve was generated from levels of standard absorbance minus the background absorbance of the glucose reagent alone. The absorbance levels from water alone were subtracted from the absorbance levels for fly and water, leaving the level of fly background for each sample. This value was then subtracted from each individual sample as ‘background’. The absorbance value of the glucose reagent alone (without fly sampled added) was subtracted from each sample, leaving the levels of glucose, and glucose released from trehalose or glycogen per sample. The addition of trehalase, allows for the measurement of glucose released from trehalose, a major fly sugar. The addition of amyloglucosidase allowed for the measurement of glycogen, the polymeric form of stored glucose, also present in each of the fly samples. The quantities (in µg) of glucose, glycogen and glucose released from trehalose were calculated from the standard curve equation, which was produced from the glucose standards. Each experiment included at least five samples, each containing three flies, per genotype and the assay was repeated at least three times.

2.16.6. Statistical significance and error bars

All error bars included in the experimental figures are represented as standard error of the mean (SEM), as stated in the figure legends. Statistical significance stars indicate the p values being less than or equal to the following; *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. Any values greater than or equal to $p \geq 0.05$ were classed as non-statistically significant results.

Chapter 3

BCP and JDCP RNAi line survival screen following infection with *Listeria monocytogenes* and *Francisella novicida*

3.1. Introduction

Epigenetic modifications are important for normal development and disruption to these changes can lead to unwanted alterations in gene expression (Handy et al., 2011). In recent years, these modifications have been linked to a number of diseases including cancer and autoimmune disease (Bayarsaihan, 2011). The changes in gene expression observed when epigenetic modifications occur are, in most cases, due to alterations in the structure of chromatin, making the DNA more or less accessible (Narlikar et al., 2002). Epigenetic modifications including DNA methylation or histone modifications affect gene regulation by repressing or enhancing the binding of transcription factors (Moore et al., 2013).

The bromodomain is a 110 amino acid motif, originally discovered in *Drosophila* as an important chromatin-modifying factor (Tamkun et al., 1992). The bromodomain sequence is highly conserved between yeast, *Drosophila* and humans (Haynes et al., 1992). In *Drosophila*, the bromodomain has been shown to play a wide range of functions in histone modification, chromatin remodelling and as transcriptional activators or repressors (Denis et al., 2010). A range of proteins have been shown to contain bromodomains and serve a range of functions in *Drosophila. female sterile (1) homeotic* (*fs(1)h*), the only bromodomain and extraterminal domain (BET) family member in *Drosophila*, is thought to be most closely related to *Brd2* and *Brd4* in mammals (Belkina and Denis, 2012; Berkovits and Wolgemuth, 2013). Functionally, *fs(1)h* has transcriptional repression functions that are essential for proper differentiation in the early embryo (Denis et al., 2010; Florence and Faller, 2008). Testis-specific bromodomain-containing protein, tBRD-1, in *Drosophila* is expressed in primary spermatocytes, is essential for male fertility (Theofel et al., 2014) and is required for proper differentiation of the immature male sex cells known as spermatids (Leser et al., 2012). tBRD-1 acts as a reader of acetylated residues of histones at the promoters of unknown target genes (Leser et al., 2012). Bromodomain and WD repeat domain-containing protein 3 (BRWD3), which contains tandem bromodomains, has been genetically linked to the Jak/STAT pathway in *Drosophila* although little else is known about its biological function (Müller et al., 2005).

Post-translational modification of histones are essential for cellular regulation, for example, acetylation of histone residues plays a key role in the regulation of transcription (Zhao et al., 2010). The addition of an acetyl group to lysine residues leads to the neutralisation of the charge, which impacts on protein-protein interactions and protein conformation causing differences in protein assembly and enzyme activity (Kouzarides, 2000; Muller et al., 2011). Bromodomain-containing proteins (BCPs) are

well-characterised regulators of transcription and well-known epigenetic modifiers (Denis et al., 2010). The bromodomain (BD), originally described in the *Drosophila* brahma (BRM) protein, is able to recognise and bind acetylated lysine residues (Dhalluin et al., 1999; Tamkun et al., 1992). Bromodomains bind acetylated lysines, predominantly in histone tails, to facilitate the recruitment of transcription factors and other chromatin-binding proteins (Wu and Chiang, 2007). This recognition of the acetylated lysine residues by the bromodomain generally leads to transcriptional activation, and due to this, BCPs are associated with a wide range of biological processes (Berkovits and Wolgemuth, 2013; Guan and Xiong, 2011; Houzelstein et al., 2002; Wang et al., 2009). In the human genome, there are 46 proteins that contain bromodomains with a wide variety of functions including co-regulators (Yang et al., 1996), co-activators (Venturini et al., 1999), mediators (Jacobson et al., 2000), chromatin modifying enzymes and scaffolding proteins (Xue et al., 2000). The bromodomain and extra-terminal domain (BET) family of proteins are characterised by two bromodomains (BD1 and BD2) and an extraterminal (ET) domain. BET proteins can recognise mono-, di- or tri- acetylated histones, however, BD1 has greater affinity for acetylated histone H4 and the recognition of acetylated H3 by the bromodomain has been implicated in chromatin remodelling (Padmanabhan et al., 2016). The BET family have various functional roles including transcriptional regulation, genome structure and viral replication (Weidner-Glunde et al., 2010; Wu and Chiang, 2007). In recent years, pharmacological inhibitors of the BET family of proteins, including IBET-151 and JQ1, have been used as treatment for cancer, inflammation and autoimmune disease (Bandukwala et al., 2012; Jung et al., 2015; Mele et al., 2013). However, the broader *in vivo* roles of BET proteins in metabolism and immunity are still not completely clear.

Similarly to the bromodomain, jumonji domains are also associated with chromatin modification and epigenetic regulation. Unlike other histone modifications, which are usually transient, methylation was thought to be a permanent modification, until the recent discovery of the first demethylases (Shi and Tsukada, 2013). Histone methylation states are dynamically regulated by a number of different groups of methyltransferases and demethylases (Bannister and Kouzarides, 2011; Shi and Whetstine, 2007). Much like other histone modifications, histone demethylation plays important roles in regulating gene expression and cell fate, therefore the methylation status of histones needs to be tightly controlled (Dambacher et al., 2010). Jumonji domain-containing proteins (JDCPs) play an important role in the removal of methyl groups from histones (Klose et al., 2006). The Jumonji N (JmjN) and Jumonji C (JmjC) domains are two domains that have been identified in the jumonji family of

transcription factors. It was originally suggested that the JmjN and JmjC domains always occur together, however the JmjC domain was later found without a JmjN domain in many organisms from bacteria to humans (Tsukada et al., 2006). The catalytic JmjC domain is a family of histone demethylases (KDMs) encoded by 30 genes in humans (Klose et al., 2006; Tsukada et al., 2006). The highly conserved JmjC domain is the largest class of demethylase enzymes that catalyse lysine demethylation of histones through a reaction that requires iron and α-ketoglutarate (Xiang et al., 2007b). The JmjC domain-containing histone demethylase can remove mono-, di- or tri-methylated states in histones (Klose et al., 2006). JmjC proteins have been associated with various human diseases including cancer and developmental and neurological disorders (Klose et al., 2006), its detrimental role in demethylation would suggest these histone demethylating enzymes may regulate a wide variety of cellular processes (Cloos et al., 2008). In vertebrates, *Jumonji domain-containing-3* (*Jmjd3*) is a H3K27 demethylase that catalyses the demethylation of H3K27me2/3 (Burchfield et al., 2015). The regulation of *Jmjd3* is highly gene- and context- specific, and is involved in development, cell plasticity, the immune system, neurodegenerative disease and cancer. Interestingly, *Jmjd3* can enhance both pro- and anti-inflammatory responses involved in infection and wound healing (Salminen et al., 2014). In cytokine-stimulated human leukemic monocytic cells (THP-1), *Jmjd3* knockdown reduces the expression of key inflammatory markers (Das et al., 2013). Changes in these pro- and anti-inflammatory markers are involved in NF-κB, chemokine and CD40 signalling, and knocking down *Jmjd3* inhibits a number of NF-κB-regulated inflammatory genes (Das et al., 2010). JMJD3 also responds to bacteria, parasites and viruses; following the addition of lipopolysaccharide (LPS) to macrophages in culture, JMJD3 is recruited to the start sites of over 70% of LPS inducible genes (De Santa et al., 2007, 2009). Deletion of lysine-specific demethylase 3B (KDM3B) can induce myeloid leukemia (Kim et al., 2012) and breast cancer (Paolicchi et al., 2013), whereas deletion of KDM5D is found in 50% of prostate cancer (Perinchery et al., 2000) and mutations associated with KDM7B has been identified in individuals with autism spectrum disorder (Shalaby et al., 2017).

In *Drosophila,* much like in humans, jumonji domain-containing proteins influence gene expression and chromatin organisation by demethylating histones (Klose et al., 2006). The demethylation process plays an important role in regulating gene activity across the genome (Bannister and Kouzarides, 2011). There are 13 JmjC genes in *Drosophila*, ten of the genes do not critically regulate development (Shalaby et al., 2017). The three other genes have detrimental roles in *Drosophila* development. Little imaginal discs (*Lid*) and Utx histone demethylase (*Utx*) are

currently the best studied members of the JmjC family in *Drosophila* (Shalaby et al., 2017) However, *Utx* and *Jumonji, AT rich interactive domain 2 (Jarid2)* null mutants exhibit lethality, whilst a third mutant, *Lid,* displays semi-lethality (Gildea et al., 2000; Herz et al., 2010; Lloret-Llinares et al., 2008). Mutations in *KDM4A* and *KDM4B* interfere with transcriptional activation of the ecdysone receptor (Tsurumi et al., 2013) and mutations in different *JmjC* domain genes modify hippo signalling causing an overgrown eye phenotype (Shalaby et al., 2017) and hedgehog pathway dysregulation leading to a wing vein phenotype (Shalaby et al., 2017). Additionally, a number of JmjC genes, *JMJD5*, *JMJD7* and *NO66* have been found to function as hydroxylases, not as histone demethylases (Chowdhury et al., 2014; Ge et al., 2012).

Many aspects of innate immunity are conserved between insects and mammals. As *Drosophila* are easily manipulated genetically, they are a powerful model for studying immune function, infection and disease (Dionne and Schneider, 2008). *Drosophila* are also able to detect and discriminate between distinct classes of microorganisms (Lemaitre et al., 1997). The *Imd* pathway, activated predominantly by peptidoglycan (PGN) of Gram-negative bacteria, can control host defence against these infections (Gottar et al., 2002). Following the detection of Gram-positive bacteria and fungi, the *Toll* pathway is activated following the cleavage of *Spätzle*, the ligand for the receptor, *Toll*. *Drosophila* infections are complex and almost every microbe can cause a different type of pathology (Dionne and Schneider, 2008). As in humans, some of the tissue damage that occurs during infection is due to an over-aggressive immune response rather than the microbe itself (Dionne and Schneider, 2008).

The Gram-positive bacterium, *Listeria monocytogenes*, is an abundant, intracellular pathogen and the causative agent of a number of food-borne diseases in humans (Farber and Peterkin, 1991). In *Drosophila, L. monocytogenes* is a lethal infection that causes infection-induced anorexia (Chambers et al., 2012). The infection activates the *Toll* pathway and alters energy metabolism and the infected flies gradually lose energy stores in the form of triglycerides and glycogen (Chambers et al., 2012). Following *L. monocytogenes* infection, *Drosophila* show a reduction in metabolites and enzymes involved in the two main energy pathways, β-oxidation and glycolysis (Chambers et al., 2012). Furthermore, mutations in *dif,* the NF-κB-related factor, leads to an increased susceptibility to *L. monocytogenes* infection than that of wild-type flies (Mansfield et al., 2003).

The Gram-negative intracellular bacterium, *Francisella novicida*, is closely related to *F tularensis*, a pathogen known to cause tularaemia, a severe infection in humans characterised by ulcers, fever and weight loss (Pechous et al., 2009). Human

infection with *F. novicida* is extremely rare and the cases that do occur are usually associated with immunocompromised individuals or those with underlying health issues (Brett et al., 2012). However, *F. novicida* is highly virulent in mice and other rodents (Sjödin et al., 2012). When *Drosophila* are infected with F *tularensis,* a highly virulent species closely related to *F. novicida,* the bacteria resided both inside hemocytes and extracellularly in the open circulatory system (Vonkavaara et al., 2008). The production of AMPs during *Francisella* infection is shown to be under the control of the *Imd* pathway (Vonkavaara et al., 2008) and the *Imd* pathway, not the *Toll* pathway, is also essential for combating *F. novicida* infections (Moule et al., 2010). In this study *Drosophila* were infected with *F. novicida* strain U112, a wild type strain that causes virulent infections in its natural mouse and rabbit host but it is not pathogenic to humans (Moule et al., 2010).

Little is known about the roles of bromodomain-containing proteins (BCPs) or jumonji domain-containing proteins (JDCPs) in the regulation of immunity or metabolism in *Drosophila*. In order to identify the roles of BCPs and JDCPs in immunity, we performed an RNAi survival screen knocking down each of the described BCPs or JDCPs in the fat body or hemocytes of *Drosophila* (see Table 2-2). Following tissue-specific knockdown, the flies were infected with *Listeria monocytogenes* or *Francisella novicida* (Ahlund et al., 2010; Mansfield et al., 2003). Both *L. monocytogenes* and *F. novicida* were injected directly in the body cavity, bypassing epithelial barriers and the gut, allowing us to focus on systemic infection. For each RNAi knockdown, two fat body drivers and two hemocyte drivers were used to test the validity of the tissue-specific drivers and the reproducibility of the results.

3.1.1. Objective and aims

The objective of this chapter is to identify whether BCPs or JDCPs have important roles in the fat body or hemocytes following bacterial infection.

The aims of this chapter are:

1. To knockdown each of the known BCPs and JDCPs in *Drosophila* using RNAi lines and tissue-specific driver lines for the fat body or hemocytes.
2. To infect each of the knockdown lines with *Listeria monocytogenes* or *Francisella novicida* and record survival effects.

3.2. Survival screen following bacterial infection using fat body drivers

The fat body, an organ analogous to adipose tissue and the liver in mammals, is the main site of the humoral immune response and metabolic homeostasis (Arrese and Soulages, 2010). Following infection, the fat body produces antimicrobial peptides (AMPs) via two immunoregulatory pathways, known as *Toll* and *Imd* to help *Drosophila* defend themselves (De Gregorio et al., 2002). The *Toll* pathway is activated by the detection of Gram-positive bacteria and fungal glycans, whereas the *Imd* pathway is usually mediated by the detection of Gram-negative peptidoglycan (Hoffmann, 2003). To investigate the role of BCPs or JDCPs in the fat body, we used two fat body specific drivers, *c564-Gal4* and *r4-Gal4* to knockdown each of the RNAi lines.

3.2.1. *c564-Gal4*

c564-Gal4 is a *Gal4*-driver line expressed in the fat body, salivary glands, imaginal discs, gut and brain, along with the lymph gland of *Drosophila* larvae (Harrison et al., 1995).

In the survival screen performed, flies that showed a median survival time either two or more days longer or shorter than the driver only control (*c564-Gal4* crossed to w^{1118}; *c564>0*) were considered to show a biologically significant survival phenotype following bacterial infection in fat body knockdowns using *c564-Gal4* (Figure 3-1). Each RNAi line, in all experiments was run at 29°C with two control groups, an uninjected group and PBS injected group as a wounding control (data not shown).

Following the knockdown of RNAi lines using *c564-Gal4*, we found one short-lived RNAi line following infection with both *L. monocytogenes* and *F. novicida*, which was KK108662. KK108662 knockdown in the fat body reduced the median survival time from both *L. monocytogenes* and *F. novicida* infections to below 2 days, whereas the driver only control showed a median survival time of 5-7 days depending on the bacteria. KK108662 is an RNAi line specific for CG2252, also known as *female sterile (1) homeotic, fs(1)h*. *fs(1)h* is a bromodomain-containing protein that plays a role as a negative regulator of transcription (Florence and Faller, 2008) and dendrite morphogenesis (Bagley et al., 2014). We also found a number of long-lived RNAi lines following infection with *L. monocytogenes* including KK107378, KK108943 and KK107819. These lines showed a prolonged median survival time of 9 days following *L. monocytogenes* infection compared to the control, which had a median survival time of 7 days. KK107378 is an RNAi line specific for *CG30417*, a *testis-specifically expressed bromodomain-containing protein-3* (*tbrd-3*). *tbrd-3* is a bromodomain-

containing protein, but little is known about its function (Theofel et al., 2014). KK108943 is an RNAi line specific for *CG4107*, also known as *Gcn5 acetyltransferase* (*Gcn5*). *Gcn5* is a bromodomain-containing protein involved in histone H3 and H4 acetylation (Guelman et al., 2006; Suganuma et al., 2008), chromatin remodelling (Suganuma et al., 2008) and muscle development (Parrish et al., 2006). KK107819 is an RNAi line specific to *CG2982,* a jumonji domain-containing protein, currently with no name or known function. The prolonged survival was not observed following *F. novicida* infection (Figure 3-1).

3.2.2. *r4-Gal4*

r4-Gal4, an insertion on the third chromosome, directs reporter gene expression specifically in the *Drosophila* fat body (Lee and Park, 2004).

In the survival screen performed, flies that showed a median survival time of two or more days longer or shorter than the driver only control (*r4-Gal4* crossed to w^{1118}; *r4-Gal4>0*) were considered to show a survival phenotype following bacterial infection in fat body knockdowns using *r4-Gal4*. Each RNAi line, in all experiments was run at 29°C with two control groups, an uninjected group and PBS injected group as a wounding control (data not shown).

Using the *r4-Gal4* driver (Figure 3-2), we observed three short-lived phenotypes following infection with *L. monocytogenes* including KK106119, GD37720 and KK108662, the latter was found to be short-lived following infection with both *L. monocytogenes* and *F. novicida*. KK106119 is an RNAi line specific to *CG17603,* also known as *TBP-associated factor 1* (*Taf1*). *Taf1* is a bromodomain-containing protein involved in histone acetylation (Mizzen et al., 1996), regulation of Jak/STAT signalling (Kallio et al., 2010), and a positive regulator of Notch signalling (Xie et al., 2014). GD37720 is an RNAi line specific to *CG5942*, known as *brahma*. *brahma* is a bromodomain-containing protein involved in histone acetylation (Tie et al., 2012), phagocytosis (Stroschein-Stevenson et al., 2005) and regulation of the immune response (Bonnay et al., 2014). After *L. monocytogenes* infection, KK108662 showed a median survival time of 5 days, whereas the control had a median survival time of 7 days. KK108662 was the only RNAi line that showed a significant reduction in median survival following infection with *F. novicida,* which was below 2 days compared to the control group, which had a median survival of 5 days. A number of RNAi lines were also long-lived following *L. monocytogenes* infection, including KK104879, KK107312, KK110158 and GD22108. KK104879 is an RNAi line specific to *CG14514*, also known as *Bromodomain-containing 8* (*Brd8*). *Brd8* plays roles in histone acetylation, histone exchange (Kusch et al., 2004) and a negative regulator of gene expression (Lu et al.,

2007). KK107312, is an RNAi line specific to *CG42799,* also known as *dikar*. *dikar* is a bromodomain-containing protein involved in *Drosophila* long-term learning (Akalal et al., 2011) and olfactory learning (Dubnau et al., 2003). KK110158 is an RNAi line specific to *CG33182*, known as *Lysine (K)-specific demethylase 4B* (*Kdm4B*). *Kdm4B* is a jumonji domain-containing protein involved in the demethylation of H3K36 and H3K9 (Lin et al., 2008; Lloret-Llinares et al., 2008). GD22108 is an RNAi line specific to *CG5383*, also known as *phosphatidylserine receptor* (*PSR*). *PSR* is a jumonji domain-containing protein shown to play roles in the negative regulation of JNK signalling and apoptotic processes (Krieser et al., 2007). There were no long-lived RNAi lines with the *r4-Gal4* driver line following *F. novicida* infection.

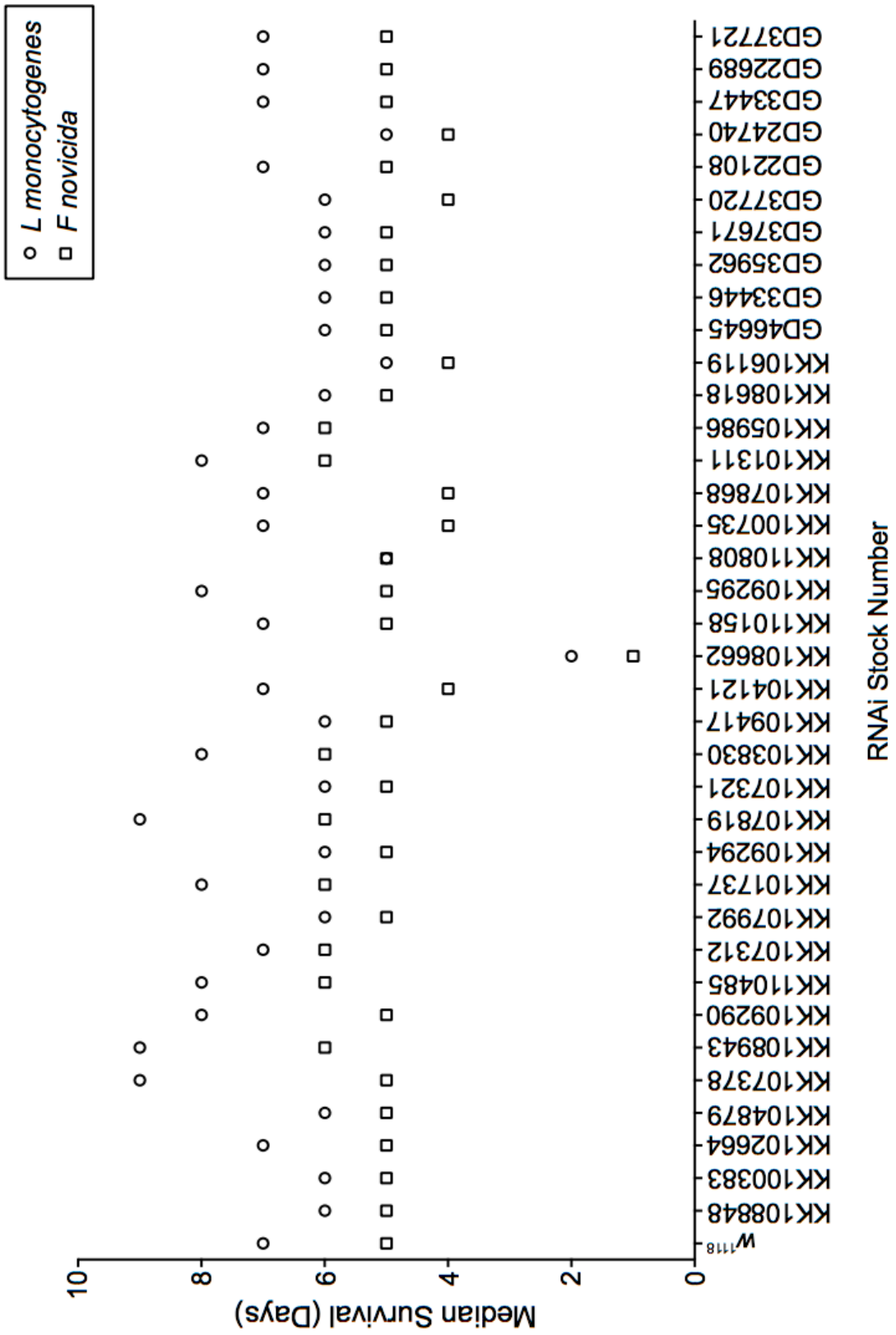


Figure 3-1: RNAi survival screen using *c564-Gal4*
5-7 day old male of each RNAi line, knockdown was driven by *c564-Gal4* in the fat body and flies were infected with *Listeria monocytogenes* (circles) and *Francisella novicida* (squares). Graph shows median survival of each RNAi line. w^{1118} was used as the control (*c564-Gal4>0*), n=20 per group.

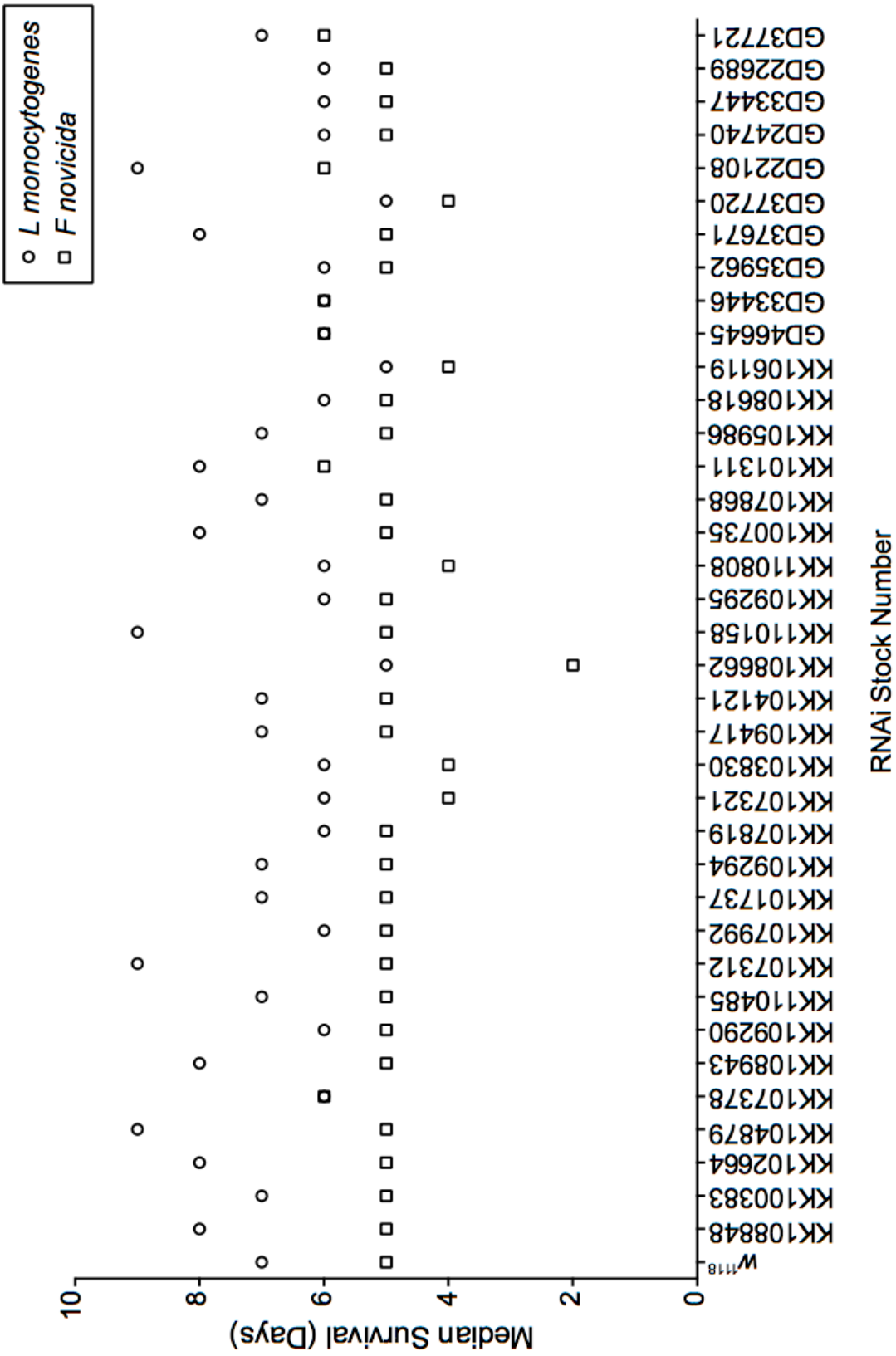


Figure 3-2: RNAi survival screen using *r4-Gal4*

5-7 day old male of each RNAi line, knockdown was driven by *r4-Gal4* in the fat body and flies were infected with *Listeria monocytogenes* (circles) and *Francisella novicida* (squares). Graph shows median survival of each RNAi line. w^{1118} was used as the control (*r4-Gal4>0*), n=20 per group.

3.3. Survival screen following bacterial infection using hemocyte drivers

The cellular arm of the *Drosophila* innate immune system is made up predominantly of hemocytes, phagocytotic cells that engulf bacteria and encapsulate large foreign particles (Lavine and Strand, 2002).

3.3.1. Hemolectin$^{\Delta}$-*Gal4*

Hemolectin (*Hml*) is a plasmatocyte specific protein involved in a number of functions including wound healing, hemolymph coagulation and hemostasis (Lesch et al., 2007). *Hml* shares homology with Von Willebrand factor (VWF), a blood-clotting factor found in mammals. The expression of *Hml* is found in both plasmatocytes and crystal cells, but has not been found in lamellocytes (Goto et al., 2003). *Hml* expression in plasmatocytes begins at stage 17 of embryogenesis and continues all the way through to adult flies (Goto et al., 2003). It has also been shown that the expression of antimicrobial peptides is not significantly affected by *Hml* RNAi (Goto et al., 2003).

The first hemocyte reporter line used in this thesis was w;*Hml$^{\Delta}$Gal4*, *UAS-2xeGFP* (Sinenko and Mathey-Prevot, 2004), it has been shown to have the greatest plasmatocyte specificity. Furthermore, *Hml$^{\Delta}$* shows robust and specific expression in the larval lymph gland and circulating hemocytes (Sinenko and Mathey-Prevot, 2004). Here, the yeast transcription factor *Gal4* is expressed under the control of an upstream *Hml* promoter sequence. These flies were also crossed with *UAS-eGFP* transgenic flies to visualise *Hml* expression with GFP if necessary (Goto et al., 2003). Flies that showed a median survival time of two or more days longer or shorter than the driver only control (*Hml$^{\Delta}$Gal4* crossed to *w*^{1118}; *Hml$^{\Delta}$Gal4>0*) were considered to show a survival phenotype following bacterial infection in hemocyte knockdowns using *Hml$^{\Delta}$Gal4* (Figure 3-3). We combined *Hml$^{\Delta}$Gal4* with each RNAi line and infected them with *L. monocytogenes* or *F. novicida.* Each RNAi line, in all experiments was run at 29°C with two control groups, an uninjected group and PBS injected group as a wounding control (data not shown).

Using the *Hml$^{\Delta}$Gal4* driver, we found three RNAi lines to be short-lived following infection with *F. novicida*; these were KK100383, KK110485 and GD33446. KK100383 is a specific RNAi line for *CG42799*, known as *dikar*, a bromodomain-containing protein. Functional information regarding *dikar* is shown above, where we also found a phenotype with another *dikar* RNAi line (KK107312) in the fat body. Even though both RNAi lines were targeting the same gene, there were no consistent phenotypes observed. KK110485 is a specific RNAi line for *CG7460*, which has no gene name or known function yet. GD33446 is a specific RNAi line for *CG1966*, also known as *ATP-dependent chromatin assembly factor large subunit* (*Acf*). *Acf* is a

bromodomain-containing protein involved in chromatin assembly (Fyodorov and Kadonaga, 2002), nucleosome assembly, mobilisation and positioning (Eberharter et al., 2001; Ito et al., 1997, 1999) and is also a negative regulator of transcription (Liu et al., 2008). All three of these lines showed a reduced median survival time of 4 days compared to the median survival time of the controls at 6 days following *F. novicida* infection. However, these lines showed no phenotype with *L. monocytogenes* infection. We also found a number of long-lived RNAi lines following *L. monocytogenes* infection; these were KK109294, KK107819, KK108662, KK109295 and KK100735. KK109294 is a specific RNAi line for *CG43320*, a jumonji domain-containing protein, with no gene name or functional information yet. As described above, KK107819 is a specific RNAi line for *CG2982,* which is also a jumonji domain-containing protein, with no gene name or known function and KK108662 is specific for *fs(1)h* and its functional information is found above. Interestingly, when we used the *Hml$^{\Delta}$Gal4* driver we found a long-lived phenotype with the *fs(1)h* RNAi line following *L. monocytogenes* infection, but not following *F. novicida* infection. KK109295, a specific RNAi line for *CG11033,* also known as *Lysine (K) specific demethylase 2* (*Kdm2*) is a jumonji domain-containing protein involved in histone H3K36 demethylation (Lagarou et al., 2008), histone H3K4 demethylation, specifically trimethyl-H3K4 (Kavi and Birchler, 2009) and histone H2A ubiquitination (Lagarou et al., 2008). KK100735, a specific RNAi line for *CG10897,* also known as *toutatis* (*tou*), is a bromodomain-containing protein involved in ATP-dependent chromatin remodelling (Emelyanov et al., 2012) and nervous system development (Vanolst et al., 2005). Even though these five RNAi lines were shown to be long-lived following *L. monocytogenes* infection, we did not detect the same phenotype following *F. novicida* infection.

3.3.2. *Croquemort-Gal4*

Croquemort (*crq*), a member of the CD36 superfamily, is expressed by hemocytes and in the gut during larval and pupal development of *Drosophila* (Evans et al., 2014). In mammals, CD36 is a membrane glycoprotein found on platelets, phagocytes, adipocytes, monocytes and some epithelia (Silverstein and Febbraio, 2009). It functions as a scavenger receptor on phagocytic cells, which recognise and eliminates foreign intruders, such as bacteria, parasites and viruses, during infection (Platt et al., 1999). It is a negative regulator of angiogenesis (Dawson et al., 1997) and due to this, it plays roles in inflammation, wound healing and tumour growth (Silverstein and Febbraio, 2009). CD36 inhibits growth factor-induced angiogenic signals mediating cell proliferation and migration (Chu et al., 2013). *Drosophila crq* is a CD36 ortholog, present on hemocytes and is involved in the recognition, engulfment

and promoting the clearance of apoptotic cells by embryonic plasmatocytes (Franc et al., 1999). It is also involved in phagosome maturation for the clearance of neuronal debris (Han et al., 2014). Much like *hemolectin, crq* is expressed on plasmatocytes through the lifespan of *Drosophila* (Guillou et al., 2016). Therefore, we used w;;*crq-Gal4,* where *crq* is under the control of *Gal4*, as a second hemocyte driver line for the survival screen in this study. Flies that showed a median survival time of two or more days longer or shorter than the driver only control (*crq-Gal4* crossed to w^{1118};;*crq-Gal4>0*) were considered to show a survival phenotype following bacterial infection in hemocyte knockdowns using *crq-Gal4* (Figure 3-4). Each RNAi line, in all experiments was run at 29°C with two control groups, an uninjected group and PBS injected group as a wounding control (data not shown).

Using the *crq-Gal4* driver, we observed no long-lived phenotypes in any of the RNAi lines with either *L. monocytogenes* or *F. novicida* infection. We found a number of short-lived lines following *F. novicida* infection with the Hml^{Δ}*-Gal4* driver, but we were unable to phenocopy them with the *crq-Gal4* driver. However, we did find a number of RNAi lines to be long-lived following *L. monocytogenes* infection. These lines were: KK110485, KK108662, KK109295, KK100735, KK101311, KK105986, KK108618, GD35962 and GD37720. All of these RNAi lines showed an increased median survival time of 8 days following *L. monocytogenes* infection compared to the median survival time of 6 days in the control group. The RNAi lines identified in this survival screen, when using *crq-Gal4* have a range of functions in *Drosophila.* KK110485 is a specific RNAi line to *CG7460*, which has no name or functional information as discussed above. We previously identified KK108662 (*fs(1)h*), KK109295 (*Kdm2*) and KK100735 (*tou*) to be long-lived following *L. monocytogenes* infection with the Hml^{Δ}*-Gal4* driver and the functional information is described above. KK101311 is a specific RNAi line to *CG1845*, also known as *Bromodomain-containing protein, 140kD* (*Br140*). *Br140* has shown to play roles in histone H3 acetylation (Huang et al., 2006) and dorsal/ventral axon guidance (Berger et al., 2008). KK105986 is a specific RNAi line to *CG5640*, known as *Utx histone demethylase* (*Utx*), which is a jumonji domain-containing protein involved in the negative regulation of H3K27 trimethylation (Denton et al., 2013; Zhang et al., 2013), along with wound healing (Campos et al., 2010) and autophagy regulation (Denton et al., 2013). KK108618 is a specific RNAi line to *CG11375*, also known as *polybromo* (*PB*) or *bap180*. *PB* is a bromodomain-containing protein involved in imaginal disc-derived leg morphogenesis and is a negative regulator of chromatin silencing (Chalkley et al., 2008). GD35962 is a specific RNAi line to *CG8569*, a bromodomain-containing protein, with no gene name or known function, other than a predicted role in

chromatin binding. We also identified GD37720, a specific RNAi line for *brahma,* which has been functionally described above. We were unable to replicate the phenotypes with both hemocyte drivers in the rest of the RNAi lines.

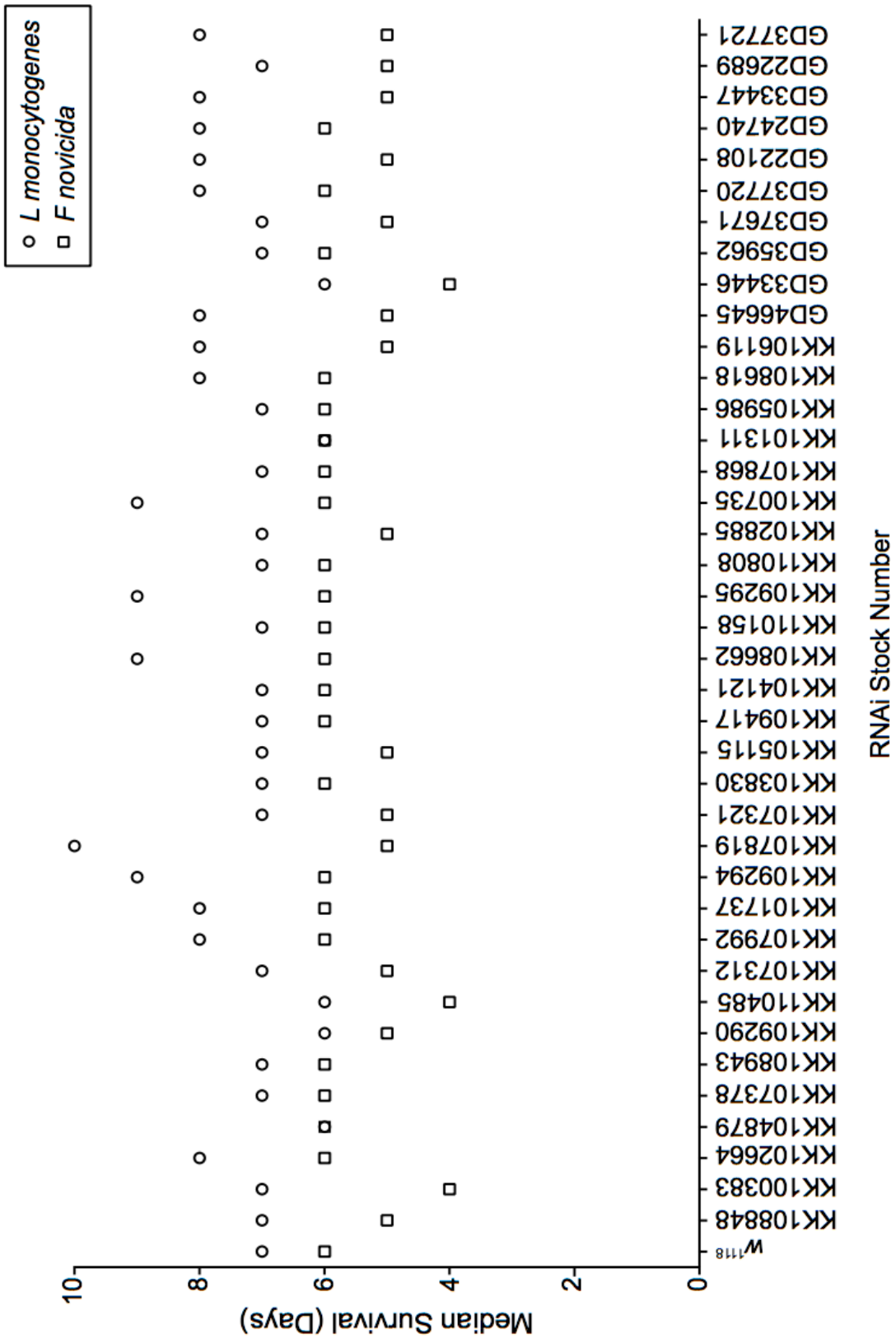


Figure 3-3: RNAi survival screen using *Hml$^{\Delta}$Gal4*

5-7 day old male of each RNAi line, knockdown was driven by *Hml$^{\Delta}$Gal4* in the hemocytes and flies were infected with *Listeria monocytogenes* (circles) and *Francisella novicida* (squares). Graph shows median survival of each RNAi line following infection. w^{1118} was used as the control (*Hml$^{\Delta}$-Gal4>0*), n=20 per group.

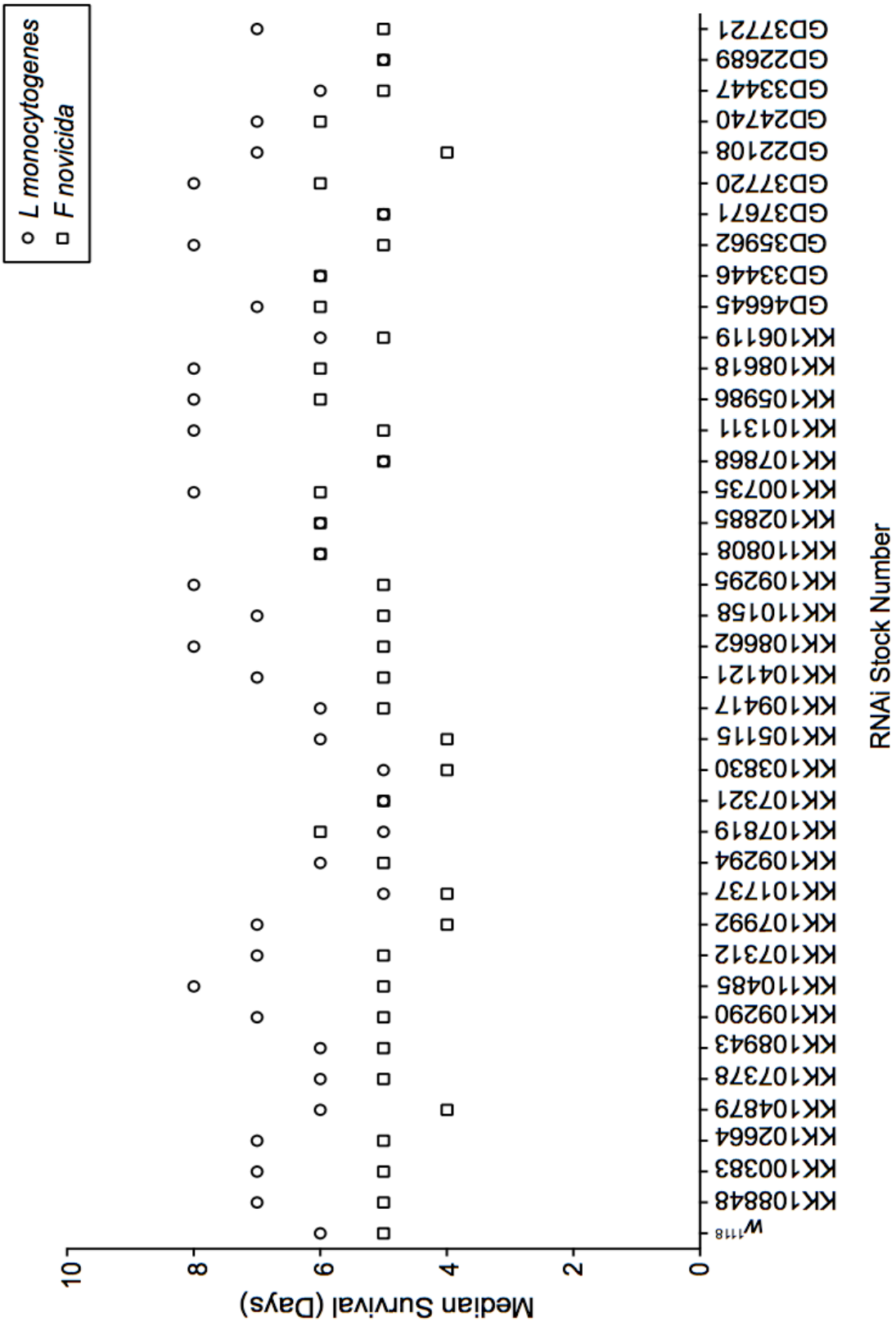


Figure 3-4: RNAi survival screen using *crq-Gal4*

5-7 day old male of each RNAi line, knockdown was driven by *crq-Gal4* in the hemocytes and flies were infected with *Listeria monocytogenes* (circles) and *Francisella novicida* (squares). Graph shows median survival of each RNAi line. w^{1118} was used as the control (*crq-Gal4>0*), n=20 per group.

3.4. Chapter 3 overview

Bromodomain-containing proteins (BCPs) and jumonji domain-containing proteins (JDCPs) both play important roles in chromatin remodelling and transcriptional modifications (Zhang et al., 2016a). However, the roles they play in *Drosophila* immunity are currently relatively unknown. To evaluate if they play a role in immunity and defence mechanisms against bacterial infection, we knocked down each of the known BCPs or JDCPs in the immune cells of *Drosophila.* There are two main types of immune cells in the fly that make up both the cellular and the humoral defence following infection (Buchon et al., 2014). The cellular defence is reliant on the hemocytes, similar to monocytes and macrophages in mammals; they play important roles in identifying and phagocytosing invading microorganisms (Vlisidou and Wood, 2015). The fat body makes up the humoral response in the fly, analogue to the mammalian adipose tissue and the liver; it produces the bulk of antimicrobial peptides (AMPs) which are able to destroy invading pathogens (Imler and Bulet, 2005).

We carried out a survival screen looking into the roles BCPs and JDCPs may have during bacterial infection, the initial screen led to the identification of a number of BCP and JDCP knockdowns in the fat body or hemocytes that were short-lived or long-lived following bacterial infection with *L. monocytogenes* or *F. novicida* (Table 3-1). Using RNAi lines for BCP or JDCP knockdowns in the fat body specifically, with the two driver lines *c564-Gal4* or *r4-Gal4*, we identified three RNAi lines (*Gcn5*, *tbrd-3* and CG2982) to be long-lived following *L. monocytogenes* infection when using the *c564-Gal4* driver. However, this long-lived phenotype was not shown for these three lines using the second fat body driver (*r4-Gal4*). In addition, we identified four lines (*brd8*, *dikar*, *Kdm4B*, *PSR*) to be long-lived following *L. monocytogenes* infection when using the *r4-Gal4* driver, but these observations were not phenocopied by the initial fat body driver. Also using the *r4-Gal4* driver, we identified two RNAi lines (*Taf1* and *brahma*) to be short-lived following *L. monocytogenes* infection. We also identified one RNAi line (KK108662, *fs(1)h*) that was short-lived following infection with both *L. monocytogenes* and *F. novicida*, when the line was knocked down specifically in the fat body using both drivers (*c564-Gal4* and *r4-Gal4*).

Following knockdown of BCPs or JDCPs in the hemocytes, using *Hml$^{\Delta}$-Gal4*, a number of RNAi lines were shown to live longer post-infection with *L. monocytogenes* than the control flies (*Kdm2*, *tou*, CG43320 and CG2982). Additionally, we observed a reduction in survival in three of the RNAi lines (*dikar*, *Acf*, and CG7460) tested following infection with *F. novicida*, however these results were not shown using the second hemocyte driver, *crq-Gal4.* Using the *crq-Gal4* driver, we also identified a

number of long-lived RNAi lines following infection with *L. monocytogenes* (*Brd140*, *polybromo, utx*, *brahma*, CG8569 and CG8569) but these results were not repeated using the original hemocyte driver. However, knocking down *Kdm2* and *tou* in the hemocytes using both hemocyte drivers extended the lifespan following infection with *L. monocytogenes.* Interestingly, the RNAi line, KK108662 (*fs(1)h*), which was shown to be short-lived following knockdown with both fat body drivers, was longer lived following *L. monocytogenes* infection when it was knocked down in the hemocytes using both driver lines. These data together suggest that following bacterial infection, knocking down *fs(1)h* may play an important and tissue-specific role in the fat body of *Drosophila.*

Using the RNAi lines that we had identified to have a survival phenotype, we repeated each of the crosses, and carried out the infections with *L. monocytogenes* and *F. novicida* for a second time. However, although we had identified a number of RNAi lines from the initial screen, to be long-lived or short-lived following bacterial infection, there was only one RNAi line that provided a consistent result in the follow-up experiments. The RNAi line was KK108662, which is specific for *female sterile (1) homeotic, fs(1)h*. This line was extremely short-lived following bacterial infection when it was knocked down specifically in the fat body. Due to this finding, we wanted to further investigate the role of *fs(1)h* in the fat body of *Drosophila.*

Stock Number & Gene Name	*c564-Gal4*	*r4-Gal4*	*Hml$^{\Delta}$-Gal4*	*crq-Gal4*
KK108662 (*fs(1)h*)	Short (*L.m/F.n*)	Short (*L.m/F.n*)	Long (*L.m*)	Long (*L.m*)
KK107378 (*tbrd-3*)	Long (*L.m*)	/	/	/
KK108943 (*Gcn5*)	Long (*L.m*)	/	/	/
KK107819 (CG2982)	Long (*L.m*)	/	/	/
KK106119 (*Taf1*)	/	Short (*L.m*)	/	/
GD37720 (*brahma*)	/	Short (*L.m*)	/	Long (*L.m*)
KK104879 (*Brd8*)	/	Long (*L.m*)	/	/
KK107312 (*dikar*)	/	Long (*L.m*)	/	/
KK110158 (*Kdm4B*)	/	Long (*L.m*)	/	/
GD22108 (*PSR*)	/	Long (*L.m*)	/	/
KK100383 (*dikar*)	/	/	Short (*F.n*)	/
KK110485 (CG7460)	/	/	Short (*F.n*)	Long (*L.m*)
GD33446 (*Acf*)	/	/	Short (*F.n*)	/
KK109294 (CG43320)	/	/	Long (*L.m*)	/
KK107819 (CG2982)	/	/	Long (*L.m*)	/
KK109295 (*Kdm2*)	/	/	Long (*L.m*)	Long (*L.m*)
KK100735 (*toutatis*)	/	/	Long (*L.m*)	Long (*L.m*)
KK101311 (*Br140*)	/	/	/	Long (*L.m*)
KK108618 (*polybromo*)	/	/	/	Long (*L.m*)
KK105986 (*utx*)	/	/	/	Long (*L.m*)
GD35962 (CG8569)	/	/	/	Long (*L.m*)

Table 3-1: RNAi lines showing a phenotype following bacterial infection
A summary of the survival phenotypes (short or long-lived compared to the *Gal4* controls) observed following infection with *Listeria monocytogenes* (*L.m*) or *Francisella novicida* (*F.n*).

Chapter 4

The immune and metabolic roles of *female sterile (1) homeotic* in the *Drosophila* fat body

4.1. Introduction

The *female sterile (1) homeotic* gene was isolated based on its embryonic phenotypes. These phenotypes include maternal mutations that cause segmental defects or deletions and thoracic homeotic transformation, while zygotic mutations cause lethality at larval or pupal stages or female sterility (Florence and Faller, 2008). *Drosophila* females lacking the function of the fs(1)h protein do not lay eggs (Perrimon et al., 1984). The homeotic defects in *fs(1)h* are similar to those caused by mutations in *trithorax* (*trx*), a key regulator of *Hox* genes (Florence and Faller, 2008). *Hox* genes are an important group of transcriptional regulators involved in vertebrate and invertebrate body planning (Mallo and Alonso, 2013). It has been shown that f*s(1)h* is critical for the activation of *Ultrabithorax* (U*bx*), a *Hox* gene that controls the differential development between wings and haltere (Weatherbee et al., 1998). Ras signalling, which affects many cellular functions including proliferation, migration, apoptosis and differentiation (Olson and Marais, 2000), has been linked with the modulation of gene expression including that of *fs(1)h* (Florence and Faller, 2008). Additionally, during early stages of embryogenesis, *fs(1)h* is required for the regulation of *Ras* targets *tailless* (*tll*) and *hückebein* (*hkb*). Furthermore, *fs(1)h* encodes a *tll* repressor, which functions to down-regulate TOR signalling (Florence and Faller, 2008).

The *female sterile (1) homeotic, fs(1)h*, gene encodes two chromatin-binding isoforms, a short form, fs(1)hS (120kDA), and long form, fs(1)hL (210kDA) (Figure 4-1) (Florence and Faller, 2008). The short isoform of *fs(1)h*, contains two bromodomains (BD1 and BD2) and an extraterminal (ET) domain at its C-terminal, which is necessary for protein-protein interactions (Lygerou et al., 1994). The long isoform is identical to the short form with an additional carboxy-terminal motif (CTM) that was shown to be essential for normal *Drosophila* development (Kellner et al., 2013). *fs(1)h* is the sole *Drosophila* member of the bromodomain and extraterminal domain (BET) family, and is homologous to mammalian bromodomain-containing proteins (BCPs), BRD2/3/4/T (Florence and Faller, 2008). However, its closest orthologues are thought to be *Brd2* and *Brd4*, which regulates transcription by recruiting Positive Transcription Elongation Factor b (PTEFb) at promoter regions of various genes in mammals (Kellner et al., 2013). Much like other BCPs, *fs(1)h* is able to recognise and bind acetylated lysine resides, as well as interact with and function as scaffolding for molecules involved in gene transcription (Sanchez and Zhou, 2009).

The fat body is analogous to adipose tissue and the liver in mammals and plays important roles in the humoral immune response and in metabolism. It produces the majority of antimicrobial peptides (AMPs) to help fight infection and control the

anabolism and catabolism of carbohydrates, lipids and sugars via a number of nutrient sensing mechanisms. Interestingly, the physiological roles of *fs(1)h* in the *Drosophila* fat body had not previously been explored.

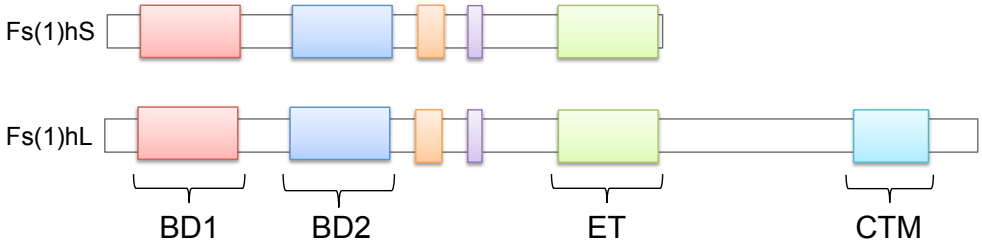


Figure 4-1: Long and short isoforms of *Drosophila fs(1)h*
Schematic of the fs(1)h proteins showing the functional modules. fs(1)hS indicates the short isoform and fs(1)hL indicates the long isoform. BD1 and BD2 are the first and second bromodomains, respectively. The C-terminal motif (CMT) is unique to fs(1)hL. ET; extraterminal domain.

4.1.1. Objective and aims

In Chapter 3, we demonstrated that the loss of *fs(1)h* in the fat body dramatically reduced lifespan following infection with *Listeria monocytogenes* or *Francisella novicida.* Interestingly, *fs(1)h* was also the only BCP in the *Drosophila* fat body to affect survival following bacterial infection. The objective of this chapter is to better understand the role *fs(1)h* is playing in the fat body of *Drosophila.*

The aims of this chapter are:

1. To further investigate the immune role of *fs(1)h* in the fat body of *Drosophila*
2. To explore the role of fat body *fs(1)h* in *Drosophila* metabolism

4.2. Confirming the knockdown of *fs(1)h* in the fat body using *c564-Gal4*

Following the RNAi survival screen and identifying that *fs(1)h* knockdown in the fat body caused the flies to be short-lived following bacterial infection (Figure 3-1 and 3-2), we firstly wanted to ensure *fs(1)h* was efficiently being knocked down by the main fat body driver, *c564-Gal4*. Gene expression analysis showed that knocking down *fs(1)h* specifically in the fat body was not detected at transcript level in whole fly samples (Figure 4-2A). However, when the transcript level of *fs(1)h* was measured in dissected fat body tissue it was significantly reduced (Figure 4-2A'). The expression of *Gal4* was also assayed and showed that *Gal4* was significantly enhanced in *fs(1)h* knockdown flies in both whole fly and dissected fat body samples (Figure 4-2B and B' respectively).

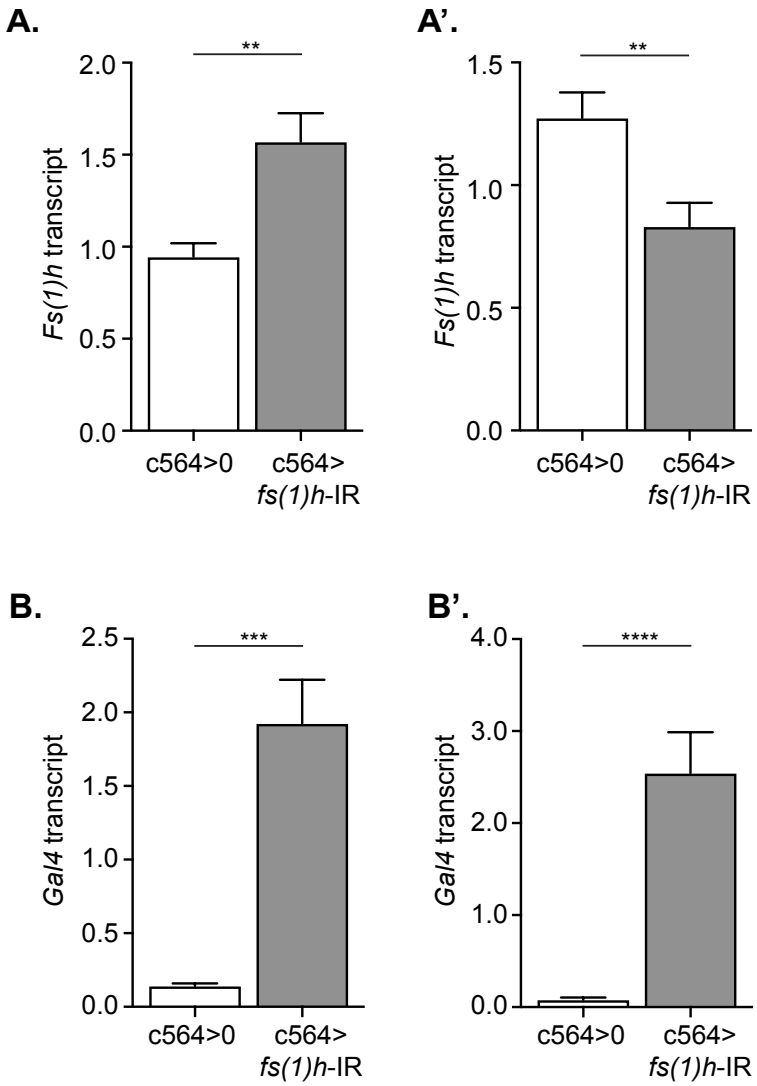


Figure 4-2: *fs(1)h* expression was reduced in dissected fat body samples following knockdown

RT-qPCR analysis of 5-7 day old fly samples following knockdown of *fs(1)h* in the fat body (*c564*>*fs(1)h*-IR, grey bars) and control samples (*c564*>0, white bars). All RT-qPCR data was normalised to the housekeeping gene, α*-tubulin*. Normalised transcript levels of **A.** *fs(1)h* expression in whole fly samples with *fs(1)h* knocked down in the fat body and controls. Unpaired t test, data shown as mean + SEM, **p<0.01 **A'.** *fs(1)h* expression in dissected fat body samples with *fs(1)h* knocked down in the fat body and controls. Unpaired t test, data shown as mean + SEM, **p<0.01, n=8-10 per genotype. **B.** *Gal4* expression in whole fly samples with *fs(1)h* knocked down in the fat body and controls. Unpaired t test, data shown as mean + SEM, ***p<0.001 **B'.** *Gal4* expression in dissected fat body fly samples with *fs(1)h* knocked down in the fat body and controls. Unpaired t test, data shown as mean + SEM, ****p<0.0001, n=8-10 biological repeats per genotype.

4.3. Knockdown of *fs(1)h* in the fat body reduced lifespan

Following the RNAi screen, experimental flies were found to be short-lived following knockdown of *fs(1)h* in the fat body. At both 25°C and 29°C and when uninfected, the flies had a severely reduced lifespan when *fs(1)h* was knocked down in the fat body using *c564-Gal4* (*c564>fs(1)h*-IR) compared to the control group. At 25°C, 50% of the *fs(1)h* knockdown flies were dead by day 8 post-eclosion (Figure 4-3A), and this was reduced further still to day 5 post-eclosion when the flies were maintained at 29°C (Figure 4-3B), whereas the control genotype (*c564*>0) showed no reduction in lifespan at 25°C or 29°C.

Following bacterial infection with *Listeria monocytogenes*, a Gram-positive intracellular bacterium, or *Francisella novicida*, a Gram-negative intracellular bacterium, *fs(1)h* knockdown flies were shown to have a further reduced survival compared to the controls (Figure 4-3C and Figure 3-1, 3-2). *fs(1)h* knockdown flies infected with *L. monocytogenes* were all dead by day 6 post-infection, whereas the control flies survived until day 9 post-infection. *F. novicida* infected *fs(1)h* knockdown flies were all dead by day 4 post-infection, unlike the controls that were all dead at day 6. The effect of *c564>fs(1)h*-IR flies also resulted in a decreased survival time following wounding, whereas the controls lived much longer. These data from the lifespan analysis and infection survivals showed that *c564>fs(1)h*-IR flies were dramatically short-lived compared to controls following infection, but also when injected with PBS, as a wounding control or completely uninjected.

To verify the specificity of the target tissue, we used a second fat body driver, *r4-Gal4*, and found the same dramatic reduction in survival following bacterial infection (Figure 3-2). The reduced lifespan under physiological conditions could also be replicated with the *r4-Gal4* fat body driver with all of the uninjected *fs(1)h* knockdown flies dead by day 10 post-eclosion, whereas the controls showed no reduction in survival (Figure 4-3D).

We can conclude from these data that the loss of *fs(1)h* in the fat body resulted in a reduction in physiological lifespan, indicating a detrimental role for *fs(1)h* in the *Drosophila* fat body. The role of *fs(1)h* during infection remains unclear as the flies were very short-lived even in the absence of infection, for this reason the role of *fs(1)h* under physiological conditions was predominantly investigated.

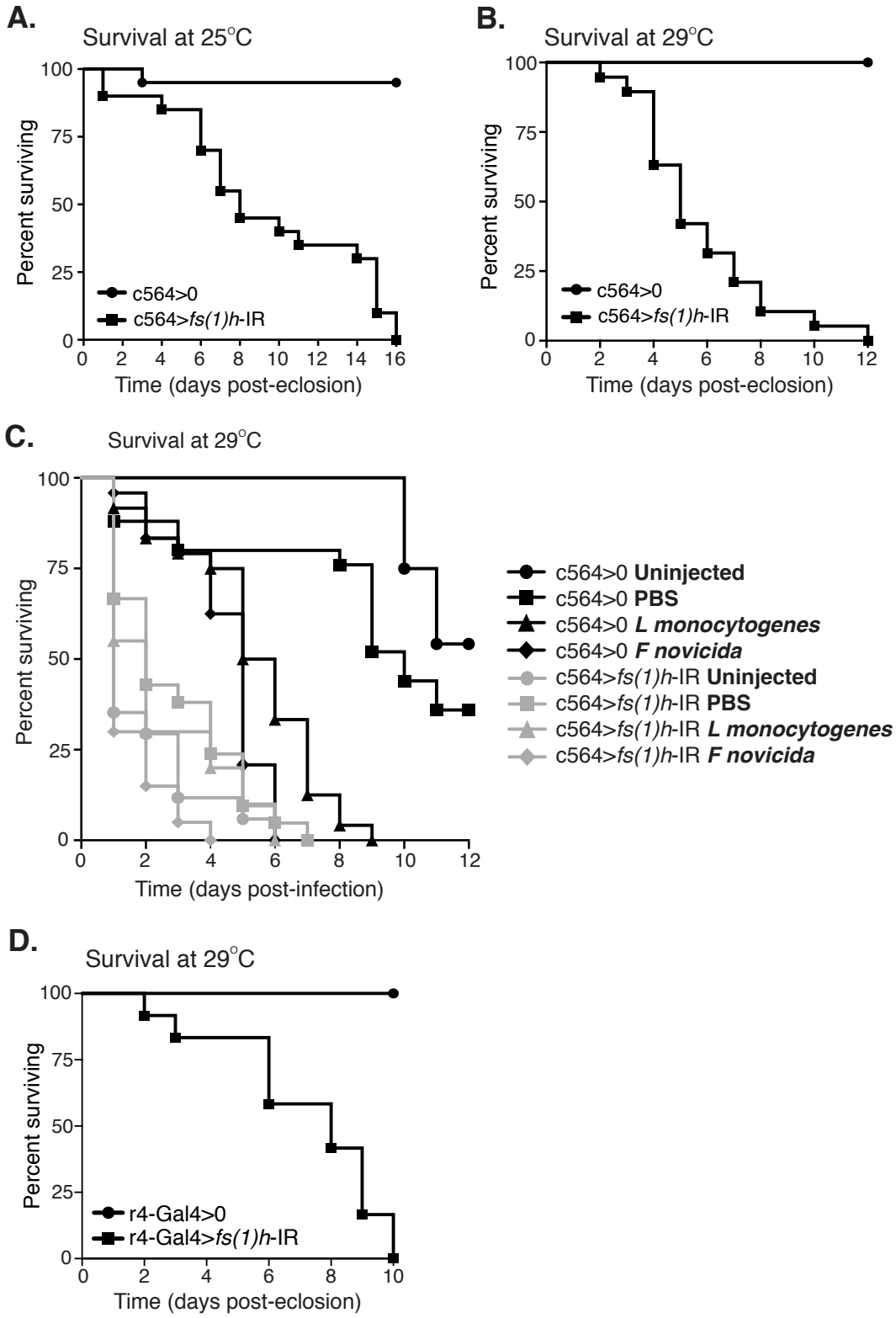


Figure 4-3: Knocking down *fs(1)h* in the fat body reduced life span

Flies were observed daily for the number of deaths and percent survival was calculated over time following knockdown of *fs(1)h* in the fat body. **A.** Survival was carried out comparing newly eclosed *fs(1)h* fat body knockdown flies (*c564>fs(1)h*-IR, squares) and control samples (*c564*>0, circles) at 25°C. n=20 per genotype. **B.** Survival was carried out comparing newly eclosed *fs(1)h* fat body knockdown flies (*c564>fs(1)h*-IR, squares) and control samples (*c564*>0, circles) at 29°C. n=20 per genotype. **C**. Survival was carried out on 5-7 day old male

flies following bacterial infection in *fs(1)h* fat body knockdown (*c564>fs(1)h*-IR, grey lines) and control samples (*c564*>0, black lines). Uninjected flies (circles), PBS injected (squares), *Listeria monocytogenes* infected (triangles, OD 0.1) or *Francisella novicida* infected (diamonds, OD 0.1) flies at 29°C, n=20 per genotype. **D.** Survival was carried out between newly eclosed *fs(1)h* fat body knockdown flies using a second fat body driver (*r4-Gal4>fs(1)h*-IR, squares) and control samples (*r4-Gal4*>0, circles) at 29°C. n=20 per genotype.

4.4. Antimicrobial peptide expression was increased following *fs(1)h* knockdown in the fat body

The fat body, an organ analogous to the liver and adipose tissue in mammals, is the principal site of the humoral response in *Drosophila* (Arrese and Soulages, 2010). Antimicrobial peptides (AMPs) are predominantly produced in the fat body and then secreted into the hemolymph (Lemaitre and Hoffmann, 2007). The AMPs are transcriptionally induced following infection by the two known peptidoglycan-responsive signalling pathways, known as the *Toll* and *Imd* pathways. Recognition of Gram-positive bacteria and fungi activate the *Toll* pathway, whereas the *Imd* pathway is triggered by the detection of Gram-negative bacteria (Lemaitre et al., 1997).

Seven AMPs produced by the *Toll* and *Imd* pathways were assayed in order to investigate the immune function of the fat body when *fs(1)h* was removed. The AMPs assayed included *Attacin A*, *Cecropin A1*, *Defensin*, *Diptericin*, *Drosocin*, *Drosomycin* and *Metchnikowin.* Transcript levels of each AMP were increased following the knockdown of *fs(1)h* in the fat body relative to the control flies (Figure 4-4 A-G). However, this transcriptional increase was only significantly upregulated compared to controls in *AttA*, *Dro* and *Drs* (Figure 4-4 A, E, F). Following fat body dissection in both *fs(1)h* fat body knockdown flies and control flies, the AMP transcript levels became increasingly upregulated compared to the control group (Figure 4-5 A-G).

Following bacterial infection with *Listeria monocytogenes* or *Francisella novicida* there was a further increase in the transcript levels of each of the seven AMP described previously when *fs(1)h* was knocked down in the fat body compared to the control group (Figure 4-6 A-G). Even though the control group showed an expected induction of AMP expression following both bacterial infections. The *fs(1)h* fat body knockdown flies (*c564>fs(1)*h-IR) showed an even more pronounced expression. Furthermore, there was no prominent induction of AMPs detected in the PBS injected control flies but *fs(1)h* knockdown flies also showed an increased AMP expression after wounding, which almost reached the expression levels of AMPs following infection of the control genotype (Figure 4-6 A-G). These data together indicated that the loss of *fs(1)h* in the fat body resulted in an upregulation of all the AMPs analysed. Therefore, *fs(1)h* may play a role as an endogenous negative regulator of AMP expression in the presence or absence of bacterial infection.

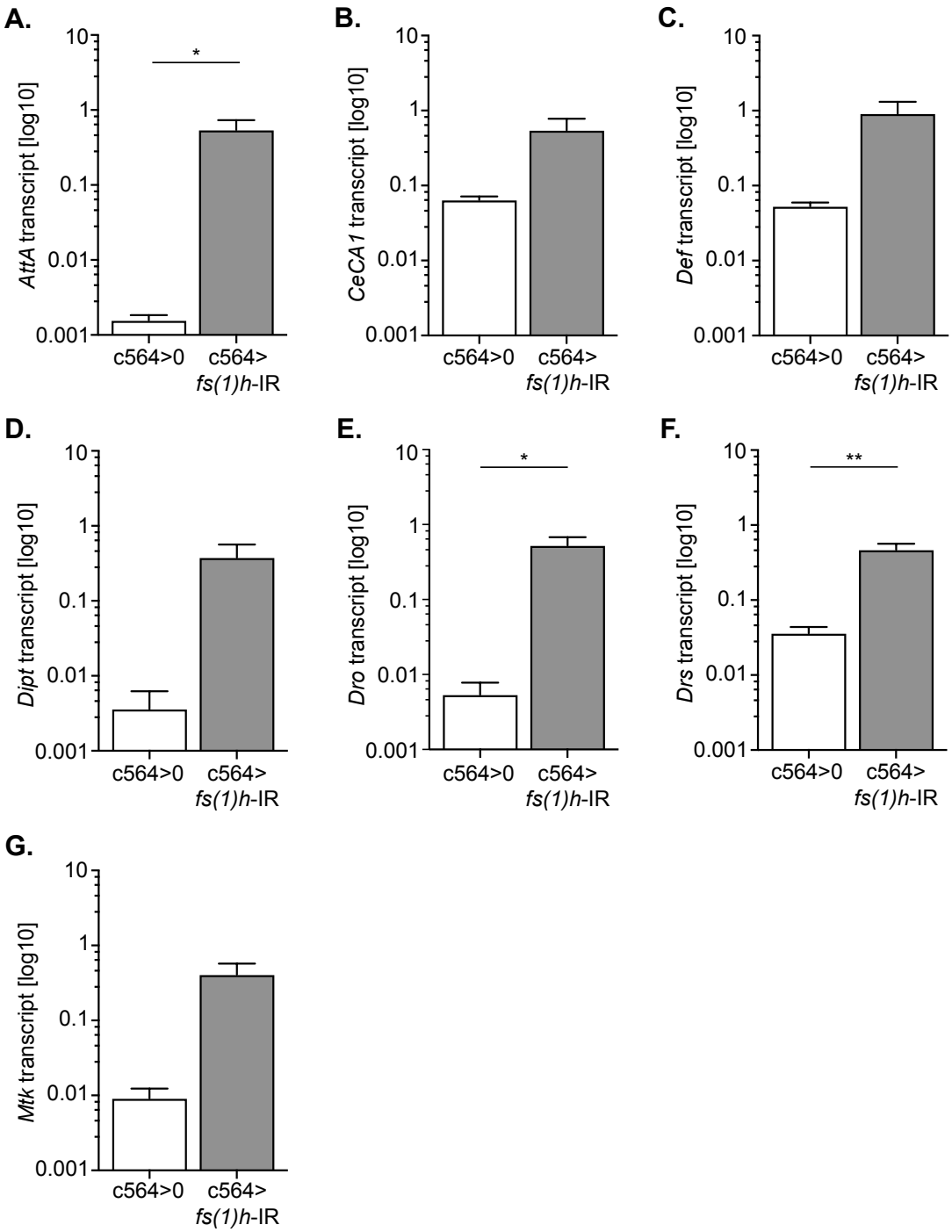


Figure 4-4: AMP transcript levels were increased following *fs(1)h* knockdown in the fat body

RT-qPCR analysis of 5-7 day old whole fly samples following knockdown of *fs(1)h* in the fat body (*c564>fs(1)h*-IR, grey bars) and control samples (*c564*>0, white bars). All RT-qPCR data was normalised to the housekeeping gene, α*-tubulin*. Data shown on a log10 scale. Normalised transcript levels of **A.** *Attacin,* **B.** *Cecropin A1,* **C.** *Defensin,* **D.** *Diptericin,* **E.** *Drosocin,* **F.** *Drosomycin* and **G.** *Metchnikowin.* Unpaired t tests, *p<0.05, **p<0.01, no stars indicate lack of statistical significance. Data represented as mean of n=8 biological repeats per genotype (3 flies per sample), error bars as SEM.

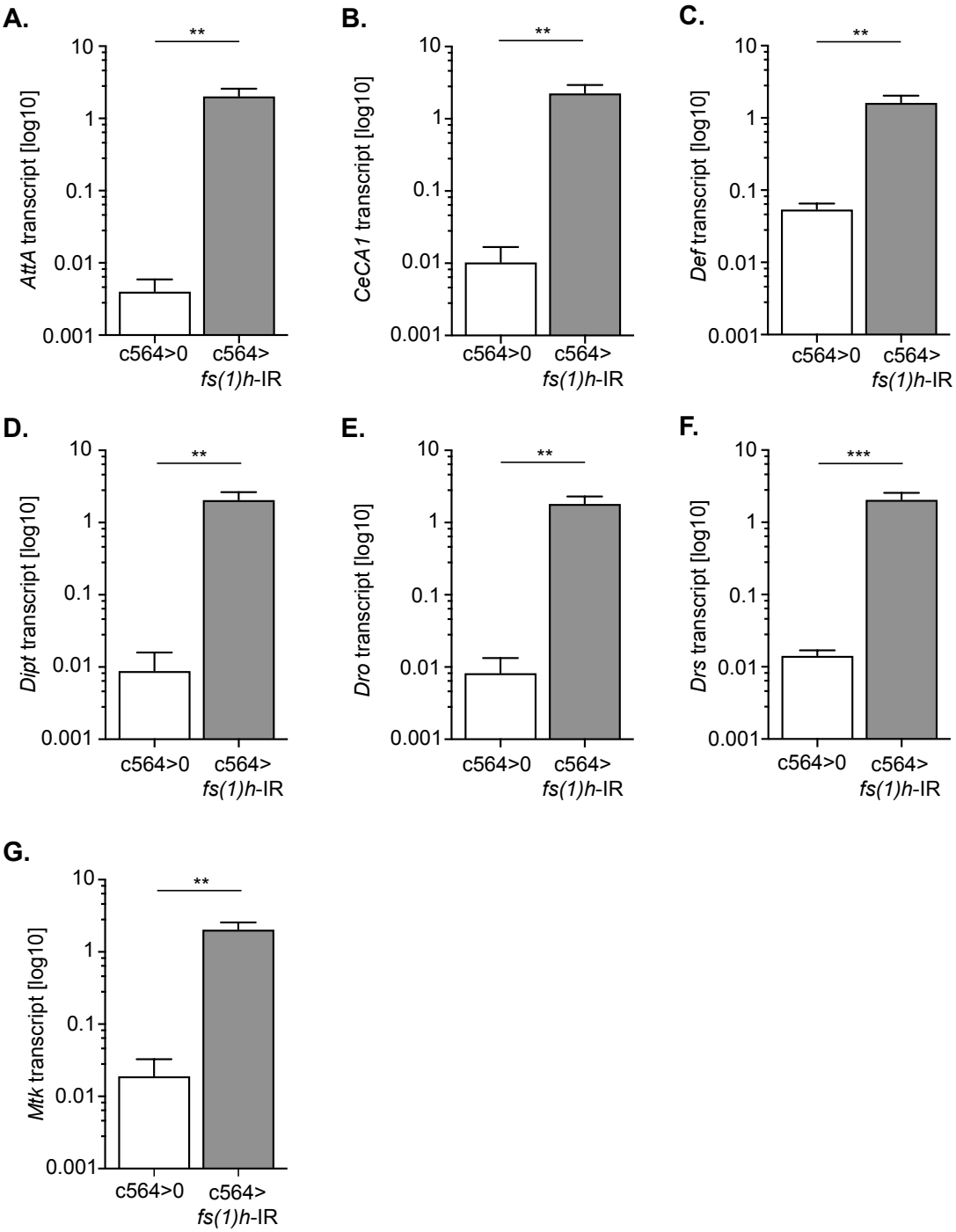


Figure 4-5: AMP transcript levels were increased in dissected fat body samples following *fs(1)h* knockdown in the fat body

RT-qPCR analysis of 5-7 day old dissected fat body samples following knockdown of *fs(1)h* in the fat body (*c564*>*fs(1)h*-IR, grey bars) and control samples (*c564*>0, white bars). All RT-qPCR data was normalised to the housekeeping gene, α-*tubulin*. Data shown on a log10 scale. Normalised transcript levels of **A.** *Attacin,* **B.** *Cecropin A1,* **C.** *Defensin,* **D.** *Diptericin,* **E.** *Drosocin,* **F.** *Drosomycin* and **G.** *Metchnikowin.* Unpaired t tests, **p<0.01, ***p<0.001. Data represented as mean of n=10 biological repeats per genotype (3 flies per sample), error bars as SEM.

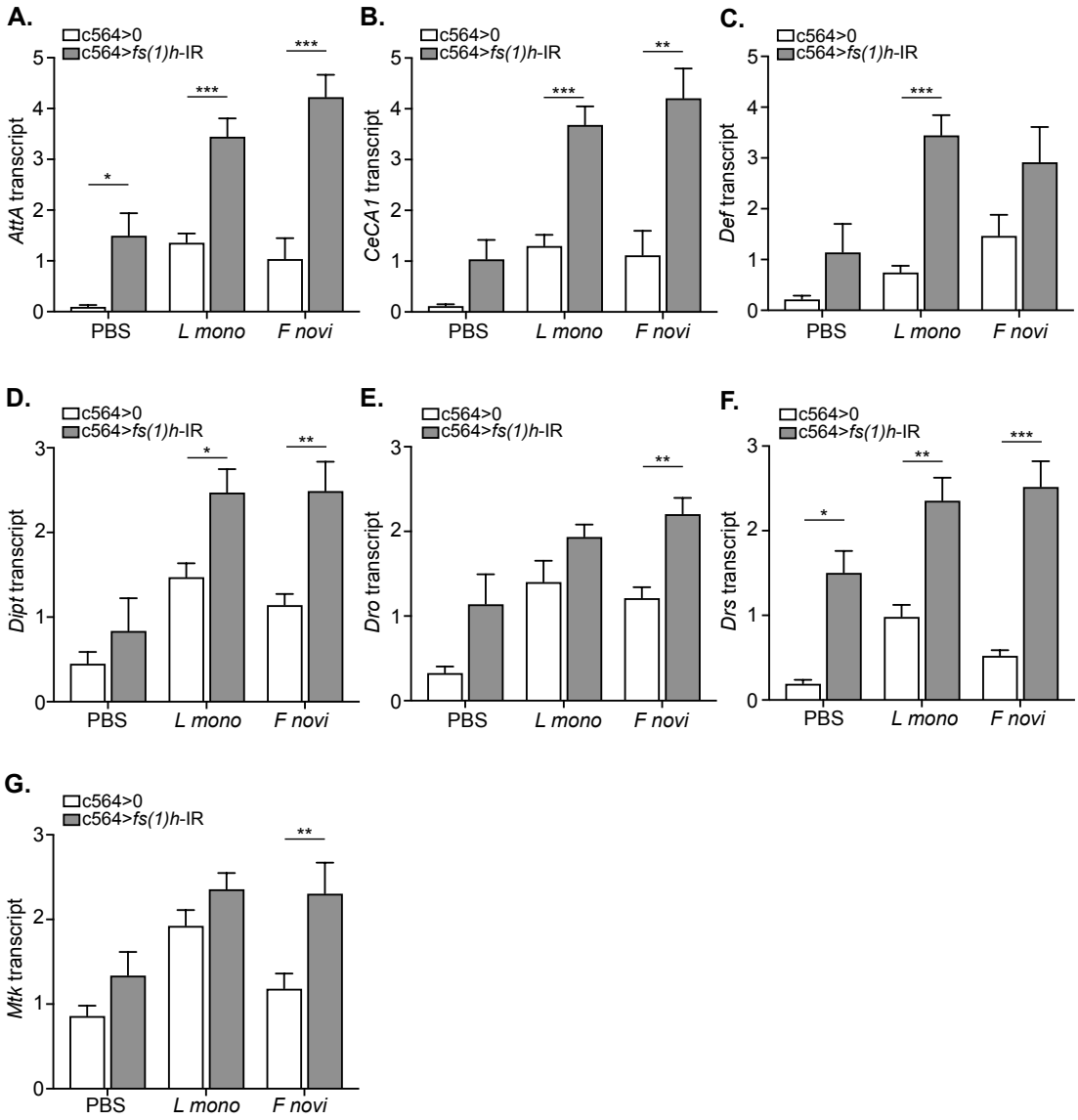


Figure 4-6: AMP transcript levels were increased following bacterial infection
RT-qPCR analysis of 5-7 day old whole fly samples following knockdown of *fs(1)h* in the fat body (*c564>fs(1)h*-IR, grey bars) and control samples (*c564*>0, white bars). Injected with PBS, as a wounding control, *Listeria monocytogenes* (*L mono*) or *Francisella novicida* (*F novi*). All RT-qPCR data was normalised to the housekeeping gene, α*-tubulin*. Normalised transcript levels of **A.** *Attacin,* **B.** *Cecropin A1,* **C.** *Defensin,* **D.** *Diptericin,* **E.** *Drosocin,* **F.** *Drosomycin* and **G.** *Metchnikowin.* Unpaired t tests, *p<0.05, **p<0.01, ***p<0.001, no stars indicate lack of statistical significance. Data represented as mean of n=5 biological repeats per genotype and injected group (3 flies per sample), error bars as SEM.

4.4.1. *Relish* showed increased protein levels when *fs(1)h* was knocked down in the fat body

NF-κB and *IκB* proteins have vital roles in the regulation of immunity and inflammation in mammals (Baldwin, 1996). Homologues to these proteins have also been shown to be conserved in *Drosophila* and play a similar role in regulating the innate immune response (Begun and Whitley, 2000). *Relish*, the *Rel/NF-κB* like transcription factor plays a key role in the *Drosophila* humoral immune response and is a key transcription factor for AMP gene expression (Hedengren et al., 1999). The activation of the *Imd* pathway, via the peptidoglycan of Gram-negative bacteria, leads to the rapid proteolytic cleavage of *Relish* into two parts by the Caspase-8 homology, Dredd (Hetru and Hoffmann, 2009; Kim et al., 2014). The N-terminal fragment, contains the DNA-binding domain of *Relish*, which can translocate to the nucleus where it can proceed to bind to *κB*-sites in DNA and drive the expression of a number of AMPs including *Drosomycin* (Hoffmann, 2003). The *IκB*-like C-terminal fragment remains in the cytoplasm following cleavage (Silverman et al., 2000). The C-terminal of *Relish* is required for endoproteolysis and signal-dependent phosphorylation by the *Drosophila* IκB kinase β (Stöven et al., 2003). *Drosophila Relish* mutants are extremely sensitive to bacterial and fungal infection and have a severe reduction in the induction of antimicrobial defence, especially the production of AMPs by the fat body (Stöven et al., 2000). Enhanced activation of the *Imd* pathway and therefore increased activity of *Relish* may result in an induction of AMP production. We detected an increased expression of AMPs in flies lacking *fs(1)h* in the fat body, and due to this, we wanted to investigate the activation of the *Imd* pathway and the transcription factor *Relish* in these flies.

Western blot analysis for total Relish protein levels showed that *fs(1)h* knockdown in the fat body led to a significant upregulation of total Relish protein when compared to the control flies (Figure 4-7A). However, the C-terminal fragment, which remains in the cytoplasm following Relish cleavage and is indicative of *Imd* pathway activation showed no change between the control and *fs(1)h* knockdown flies (Figure 4-7A). These data indicated that the increased baseline AMP expression shown previously was not due to an overall increase in *Imd* pathway activation. Interestingly, we found an accumulation of total Relish protein in flies with *fs(1)h* knockdown in the fat body, but this did not seem to be directly affecting the activation of the *Imd* pathway or explain the increased AMP expression shown. Following bacterial infection with *Francisella novicida,* there was a further increase of total Relish protein in both the PBS injected flies and in the *F. novicida* infected flies. This increase was shown slightly in the control flies, but was heightened further in the *fs(1)h* fat body

knockdown flies. The C-terminal fragment was also increased following injection with PBS or *F. novicida*, however there was no difference between the *fs(1)h* knockdown and the control flies (Figure 4-7B). These data suggested that *fs(1)h* knockdown in the fat body did not cause an increase in *Imd* pathway activation following bacterial infection. Interestingly, we found elevated total Relish protein levels in *fs(1)h* knockdown flies when uninjected, injected with PBS as a wounding control, and following bacterial infection. However, as the C-terminal did not alter following *fs(1)h* knockdown, an over-activation of the *Imd* pathway did not seem to be the reason for the elevated AMP expression or the reduction in survival following *fs(1)h* knockdown in the fat body.

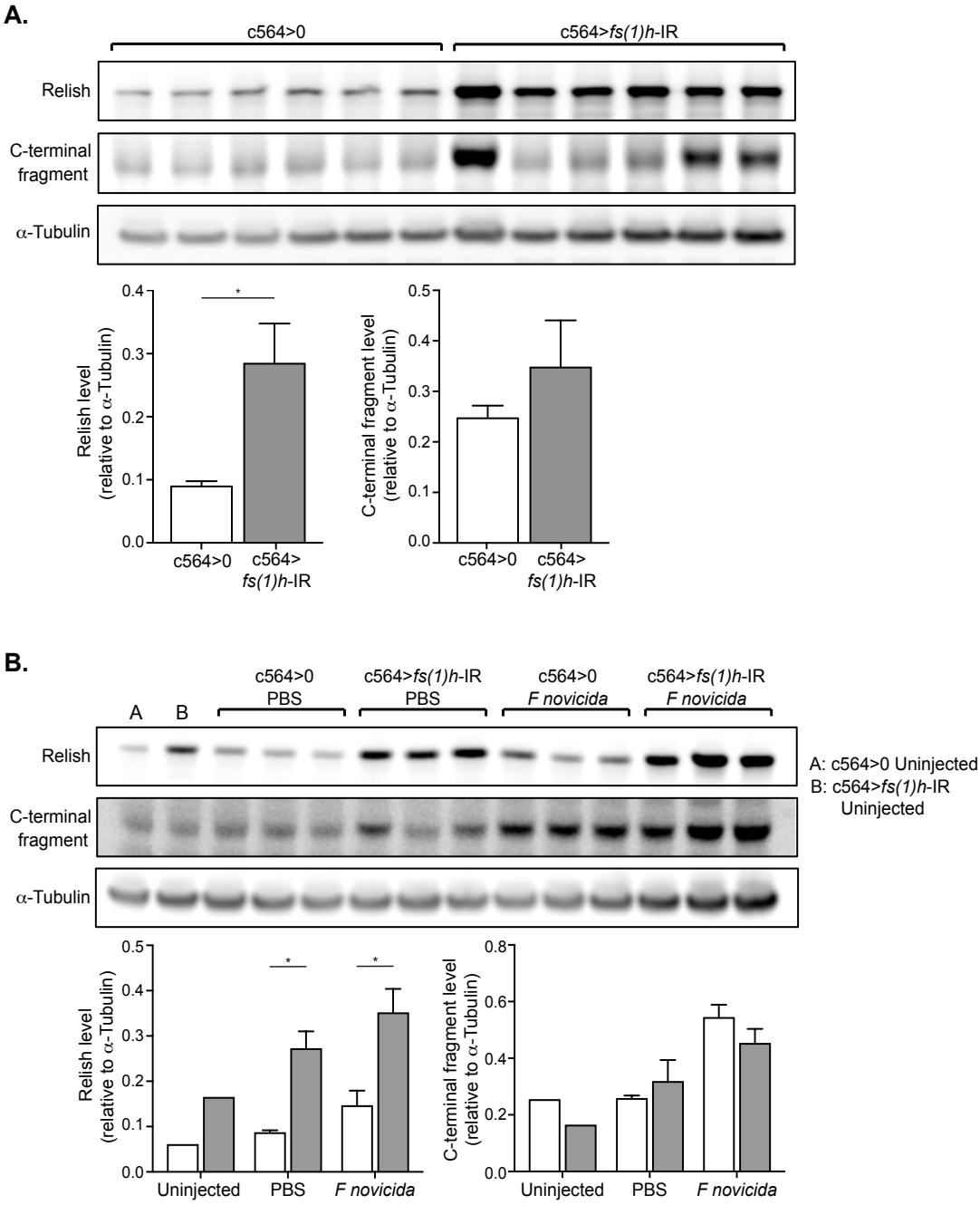


Figure 4-7: Total Relish levels were increased in *fs(1)h* fat body knockdown flies
Western blot analysis of 5-7 day old flies lacking *fs(1)h* in the fat body (*c564*>*fs(1)h*-IR) and controls (*c564*>0) **A.** Total Relish protein and cleaved C-terminal levels in *fs(1)h* knockdown flies (grey bars) and controls (white bars). Values represented as intensity relative to *α-tubulin*, data shown as mean + SEM, *p<0.05, no stars indicate lack of statistical significance. **B.** Total Relish protein and cleaved C-terminal levels in *fs(1)h* knockdown flies (grey bars) and controls (white bars), in uninjected samples and following injection with PBS or *Francisella novicida.* Data represented as mean or mean + SEM, *p<0.05, no stars indicate lack of statistical significance.

4.5. Loss of *fs(1)h* in the fat body increased cytokine expression

In addition to the production of AMPs in the humoral immune response, the fat body also releases a wide variety of immune-modulatory molecules and cytokines. Cytokines are a group of proteins that can help mediate the immune response. In *Drosophila*, cytokines have been shown to play roles in host defence, stress response and wound repair (Vanha-aho et al., 2016). Much of the cross-talk between tissues and organs in *Drosophila*, including gut-to-fat body, hemocytes-to-fat body and hemocytes-to-muscle, is carried out by cytokines that can respond to an array of stimuli and act to initiate local or systemic responses (Vanha-aho et al., 2016).

decapentaplegic (*dpp*), a TGF-β superfamily member, is homologous to the mammalian bone morphogenetic protein (BMP) and is essential for many aspects of *Drosophila* development and the immune response. *dpp* is activated by wounding and can help to resolve the antimicrobial peptide response (Clark et al., 2011). It is also an inhibitor of the immune response and inflammation following sterile injury and flies with non-functioning *dpp* exhibit strong antimicrobial peptide expression after wounding. Due to the role of *dpp* in the immune response and resolving AMP expression, the transcript levels were measured following *fs(1)h* knockdown in the fat body. We found a significant increase following knockdown of *fs(1)h* in the fat body compared to the control group (Figure 4-8A). *eiger*, the single member of the TNF superfamily, is expressed predominantly in the nervous system, is able to induce cell death by activating the JNK pathway (Igaki et al., 2002) and acts as a metabolic hormone that mediates nutrient response by remotely acting on IPCs (Agrawal et al., 2016). It also plays an important role as a key inflammatory cytokine of the immune system in *Drosophila*. *eiger* mutants are sensitive to bacterial infection, suggesting a role in the *Drosophila* innate immune response (Brandt et al., 2004). It has also been shown that *eiger* mutants are sensitive to extracellular bacteria (Schneider et al., 2007). However, following infection with intracellular bacteria the mutants respond as well as, or better than wild-type flies (Schneider et al., 2007). Following knockdown of *fs(1)h* in the fat body there was a significant upregulation in *eiger* transcript levels relative to the control group (Figure4-8B). The Jak/STAT activating ligand, *upd3*, is an important pro-inflammatory cytokine in coordinating the cellular immune response in different tissues of *Drosophila* (Yang and Hultmark, 2016). Following bacterial infection, hemocytes produce upd3, which in turn activates the Jak/STAT pathway in the fat body (Yang and Hultmark, 2016). Bacterial infection or damage to the gut can also lead to the secretion of upd3 to stimulate intestinal stem cell proliferation and differentiation (Buchon et al., 2010). Furthermore, lipid-rich diet stimulates hemocytes to secrete upd3, systemically impairing glucose homeostasis and reducing lifespan

(Woodcock et al., 2015). *upd3* levels in the fat body *fs(1)h* knockdown flies showed a significant increase relative to the control group (Figure 4-8C). Taken together, along with the increased AMP expression, we also found elevated cytokine levels following loss of *fs(1)h* in the fat body.

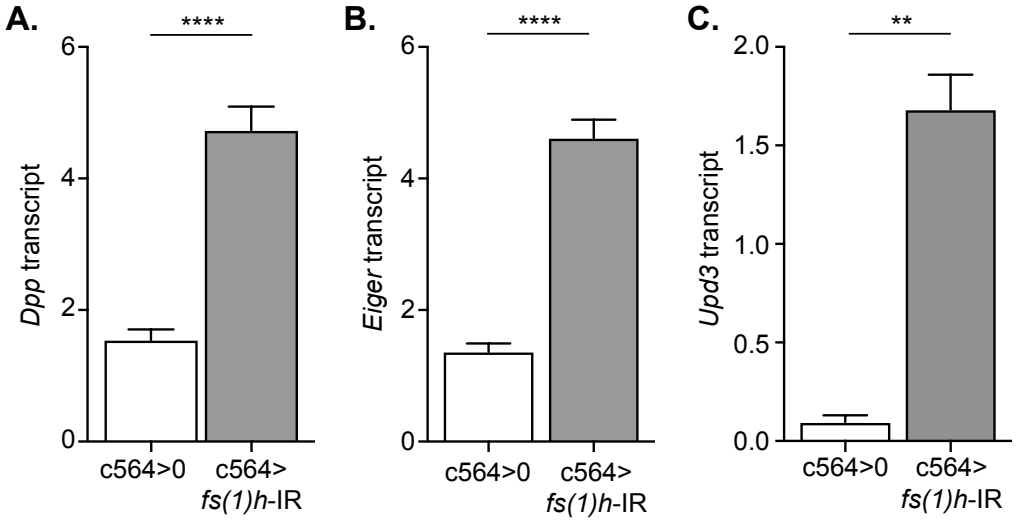


Figure 4-8: Cytokine expression was increased following *fs(1)h* knockdown in the fat body

RT-qPCR analysis of 5-7 day old whole fly samples following knockdown of *fs(1)h* in the fat body (*c564>fs(1)h*-IR, grey bars) and control samples (*c564*>0, white bars). All RT-qPCR data was normalised to the housekeeping gene, α*-tubulin*. Normalised transcript levels of **A.** *decapentaplegic (dpp),* **B.** *eiger,* **C.** *unpaired 3 (upd3).* Unpaired t tests, **p<0.01, ****p<0.0001. Data represented as mean of n=8 biological repeats per genotype (3 flies per sample), error bars as SEM.

4.6. Flies with *fs(1)h* knockdown in the fat body showed increased gut barrier dysfunction but did not alter endogenous bacterial loads

Ageing is broadly defined by a progressive decline in physiological function and this decline includes all biological systems, including molecular interactions, cellular function and tissues structure and function (He and Jasper, 2014; López-Otín et al., 2013).

The intestinal epithelium forms a barrier that allows the absorbance of nutrients and restricts contact with contaminants from the environment and microorganisms (Buchon et al., 2009). Structural and functional impairments of this epithelium have been identified in humans and *Drosophila* during the ageing process (He and Jasper, 2014; Rera et al., 2012; Saffrey, 2014). Intestinal barrier dysfunction is correlated with reduced lifespan across a number of *Drosophila* genotypes and in various environmental conditions such as dietary restriction (Rera et al., 2012). Irrespective of actual age, intestinal barrier dysfunction can be used to predict impending death in individual flies and can be used to identify flies with systemic metabolic defects including a reduction of AKT activation and FOXO upregulation (Rera et al., 2012). Remarkably, age-related increase in AMP expression is tightly linked to intestinal barrier dysfunction (Rera et al., 2012). In response to microorganisms, the reaction by the gut epithelium of *Drosophila* is complex and diverse (Buchon et al., 2013). However, gut defences include the production of reactive oxygen species (ROS), the secretion of AMPs into the hemolymph via the *Imd* pathway and epithelium renewal in response to gut damage, in order to maintain homeostasis (Kuraishi et al., 2013). Furthermore, to maintain intestinal homeostasis, commensal bacteria must be upheld, whereas pathogens must be eliminated, and inflammatory responses must be regulated (Apidianakis and Rahme, 2011). Interestingly, regulatory mechanisms such as the down-regulation of the *Imd* pathway in *Drosophila* ensures the appropriate level of immune reactivity in the gut to effectively destroy invading pathogens, but to accommodate the required gut microbiota (Buchon et al., 2013). In mammals, acute or chronic dysregulation can lead to various diseases including gastrointestinal infection and colorectal cancer (Garrett et al., 2010). The smurf assay was used to non-invasively investigate the gut integrity of flies with *fs(1)h* knocked down in the fat body. In this assay, dyed food was used to visualise the integrity of the digestive system. An intact gut does not allow the dye to leak into the body cavity of the fly, whereas a dysfunctional gut barrier allows the dye to leak into the body and the whole fly will become coloured blue. The assay showed that following *fs(1)h* knockdown in the fat body, there was a small increase in

the number of flies with gut barrier dysfunction, observed by the blue food dye being outside of the digestive tract, relative to the control group, where we did not detect any flies with dysfunctional gut integrity (Figure 4-9A). However, we did not detect gut integrity dysfunction in all the flies with *fs(1)h* knocked down in the fat body, indicating that the loss of gut integrity was not a hallmark of *fs(1)h* deficiency in the fat body, rather a secondary effect of the early death phenotype observed in these flies.

In many organisms, the microbiome is made up of extracellular bacteria and endosymbionts, which often play important roles in development, immunity and homeostasis (Elgart et al., 2016). In recent years, *Drosophila* have become an important model for studying non-pathogenic host-microbe interactions, along with the dynamics and consequences of host-microbe interaction (Chandler et al., 2011). The microbiota of the *Drosophila* gut is relatively simple compared to the complex diversity of the microbiota associated with vertebrates (Broderick and Lemaitre, 2012). Laboratory stocks of *Drosophila* are colonised predominantly by *Acetobacter* and *Lactobacillus* species (Wong et al., 2011) which influence many functions including immunity, lifespan and nutritional regulation (Ridley et al., 2012; Ryu et al., 2008; Yamada et al., 2015). Furthermore, there is an important interplay between the microbiome and the immune response, and immune deficiency or even ageing can result in increased bacterial load in the fly (Ren et al., 2007). Bacteria were identified through culture-independent assessment of microbial diversity by direct PCR amplification and sequencing of bacterial 16S ribosomal DNA extracted from *Drosophila* DNA. The amplified product for 16S ribosomal DNA showed no difference between the control flies and *fs(1)h* fat body knockdown flies (Figure 4-9B). Loss of *fs(1)h* in the fat body did not result in an increase of endogenous bacterial load compared to the control group.

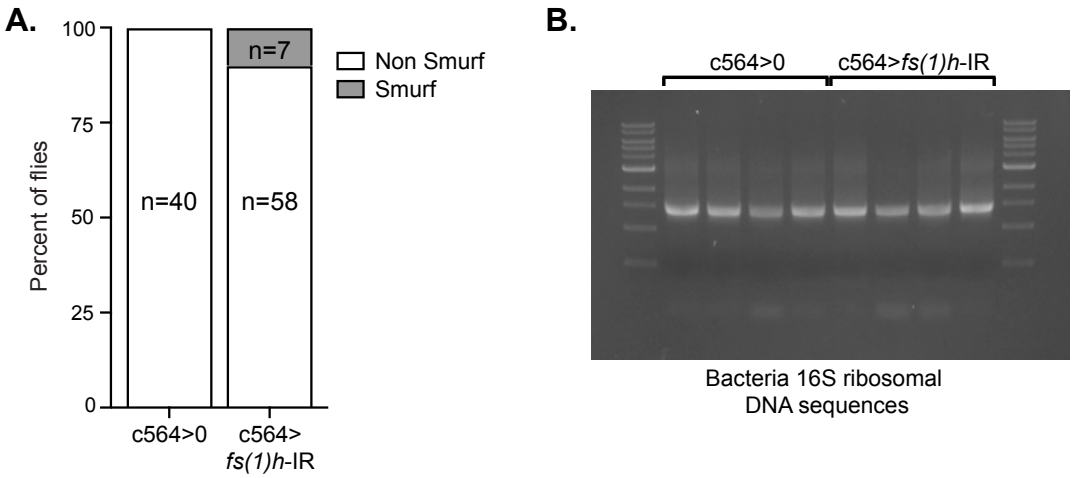


Figure 4-9: *fs(1)h* knockdown in the fat body showed a slight change in gut integrity but no change in endogenous bacterial load
Changes in the gut of controls (*c564*>0) and *fs(1)h* fat body knockdown flies (*c564>fs(1)h*-IR) were investigated. **A.** Smurf assays for the gut integrity of 5-7 day old male flies with *fs(1)h* knocked down in the fat body and controls. Data represented as percentage, non-smurfs (white bars), and smurf flies (grey bar). **B.** Amplified product for bacterial 16S ribosomal DNA in 5-7 day old male *fs(1)h* knockdown flies and controls.

4.7. Fat body knockdown of *fs(1)h* reduced survival during starvation

Drosophila are a widely studied model of starvation responses (Gibbs and Reynolds, 2012). There are three main ways to increase starvation resistance; by storing more energy, by consuming that energy at a lower rate or by tolerating the loss of the energy stores (Lee and Jang, 2014). The fat body is the main organ for the storage and mobilisation of energy, we aimed to investigate whether the loss of *fs(1)h* in the fat body affected the survival and tolerance to starvation stress. Flies with fat body knockdown of *fs(1)h* had a reduced life span on starvation food compared to the control flies (Figure 4-10). Half the *fs(1)h* knockdown flies were dead after approximately 10 hours on starvation food, whereas the control flies still had 50% survival around 40 hours. Along with the reduction in lifespan under both physiological and infected conditions we found previously, we also found a dramatically reduced tolerance towards starvation stress when *fs(1)h* was downregulated in the fat body.

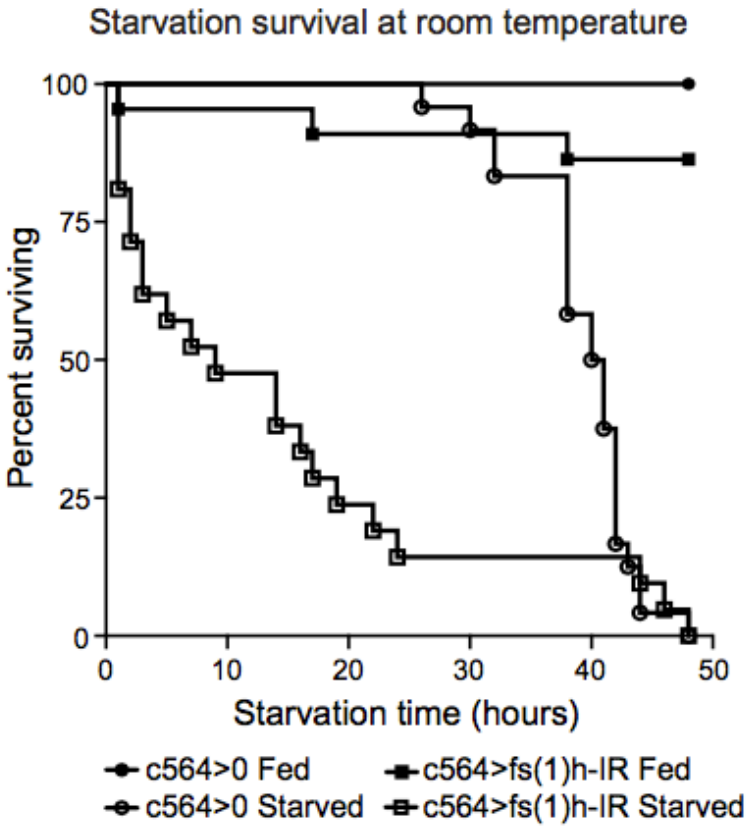


Figure 4-10: Knocking down *fs(1)h* in the fat body reduced starvation survival
Starvation survival of 5-7 day old control flies (*c564*>0, circles) and flies with *fs(1)h* knockdown in the fat body (*c564*>*fs(1)h*-IR, squares). Fed flies (closed shapes) and starved flies (open shapes). Flies were counted every two hours during the day for deaths and percent survival was calculated, n=20 per group.

4.7.1. Flies lacking *fs(1)h* in the fat body were unable to utilise their triglyceride stores during starvation

The fat body is not only important in the humoral immune response; it also plays fundamental roles in energy storage and utilisation (DiAngelo et al., 2009). Fat body cells control the synthesis and use of fat and glycogen, along with the production of many proteins and metabolites required in the hemolymph (Arrese and Soulages, 2010). The fat body is analogous to adipocytes, which are characterised by the presence of lipid droplets, made up predominantly of triglycerides (TAG). The triglycerides are produced from dietary carbohydrates, fatty acids or proteins (Arrese and Soulages, 2010). However, during times of energy demand, *Drosophila* can access their triglyceride stores via lipolysis (Arrese and Soulages, 2010). The process of lipolysis is carried out by a number of lipases working together. As there was a severe starvation phenotype in the *fs(1)*h knockdown flies, thin layer chromatography (TLC) was used to analyse triglyceride levels within the flies. These data showed that *fs(1)h* knockdown in the fat body showed no difference in the quantity of triglyceride present under normal fed conditions (Figure 4-11). However, the *fs(1)h* knockdown in

the fat body limited the ability for the flies to mobilise triglycerides following a 24-hour starvation period when compared to controls (Figure 4-11). Control flies completely emptied and utilised their triglyceride stores for energy during 24-hours starvation. Conversely, flies with *fs(1)h* knocked down in the fat body only showed a slight reduction in triglyceride levels following 24-hours starvation, suggesting they were incapable of utilising their stored triglycerides. This inability for the flies to utilise their triglyceride stores fits with the observation that they die much earlier than the control flies on starvation food. It also indicates why the *fs(1)h* fat body knockdown flies had a significant reduction in starvation tolerance and a deficiency in energy mobilisation.

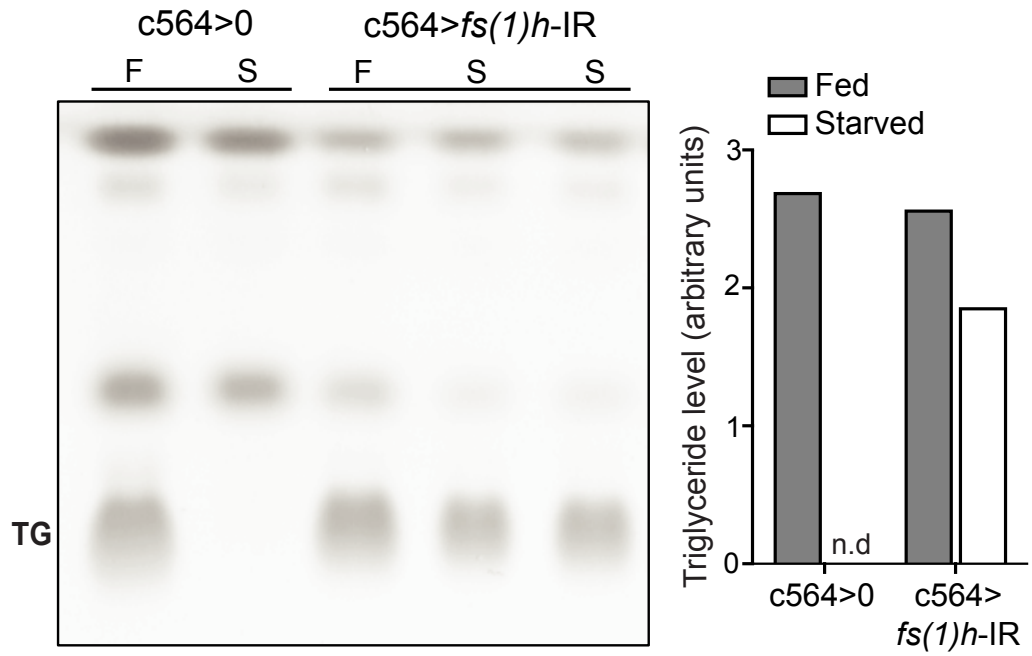


Figure 4-11: *fs(1)h* fat body knockdown flies were unable to utilise their triglyceride stores during starvation

Thin layer chromatography (TLC) analysis of 5-7 day old total fly triglyceride levels following knockdown of *fs(1)h* in the fat body (*c564>fs(1)h*-IR) compared to controls (*c564>0*). Fed conditions (grey bars) and following 24-hour starvation (white bars). The TLC plate shows one fed and one starved group for the control, and one fed and two starved groups (represented by the mean) for the *fs(1)h* fat body knockdown. F; fed, S; starved and TG; triglyceride, n.d; non-detected.

4.7.2. Morphological changes to the fat body were not observed following *fs(1)h* knockdown in the fat body

There are approximately 18,000 fat body cells in the abdomen of adult flies and the proportion of lipids and glycogen remain relatively constant throughout adult life (Johnson and Butterworth, 1985). However, dense, protein-rich granules can be seen in fat body cells of ageing flies (Johnson and Butterworth, 1985). As *fs(1)h* knockdown flies were unable to utilise their triglyceride stores during starvation, we wanted to investigate whether removing *fs(1)h* from the fat body changed the appearance or morphology of the lipid droplets. Staining the adult fat body with LipidTOX, a dye staining neutral lipids, showed no overt morphological abnormalities in the tissue following knockdown of *fs(1)h* in the fat body compared to the control group (Figure 4-12). We can conclude that under fed conditions, there were no morphological abnormalities in the fat body cells deficient for *fs(1)h.*

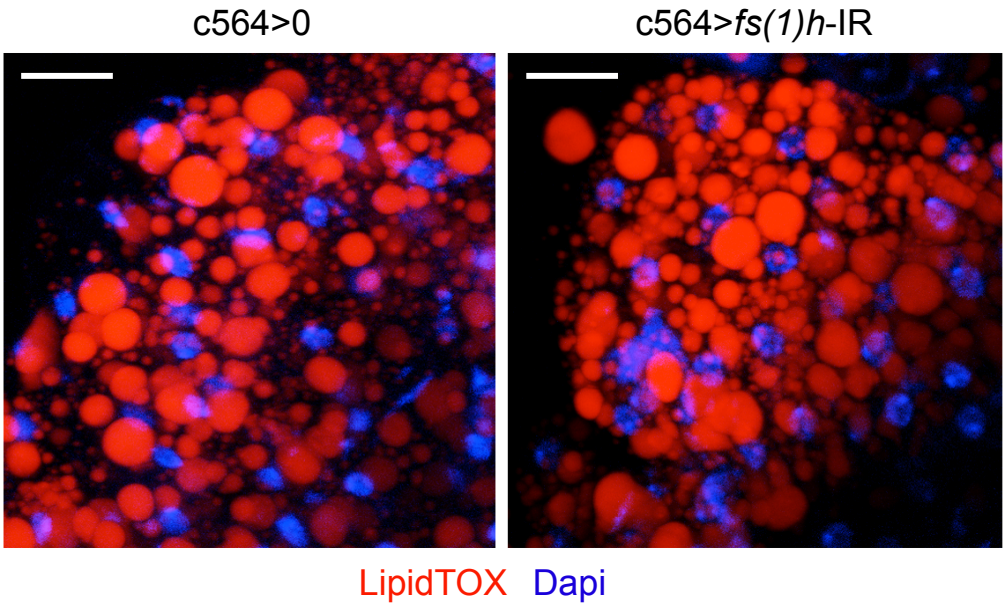


Figure 4-12: The morphology of lipids remained the same as controls following *fs(1)h* knockdown in the fat body
Isolated adult fat body tissue aged for 5-7 days from *fs(1)h* knockdown flies (*c564>fs(1)h*-IR, right) and controls (*c564*>0, left), stained with LipidTOX (red, neutral lipids) and DAPI (blue, nuclei). Scale bar, 20μm.

4.7.3. Loss of *fs(1)h* in the fat body led to a transcriptional reduction in a number of key lipid mobilisation genes

Lipases are hydrolytic enzymes that operate on the surface of lipid substrates to break down triglycerides into free fatty acids and glycerol (Pistillo et al., 1998). In *Drosophila,* and numerous other organisms, lipases control the balance between triglyceride accumulation and mobilisation (Grönke et al., 2007). There are thought to be two modes of lipolysis, firstly basal lipolysis, which balances steady-state lipogenesis in lipid storage homeostasis (Kühnlein, 2012). The second mode is stimulated lipolysis, which causes TAG to be mobilised during periods of energy shortage, for example during starvation (Kühnlein, 2012). Having identified impaired utilisation of triglyceride stores upon starvation in the *fs(1)h* knockdown flies, the transcript levels of key lipases involved in lipid metabolism were assayed.

Hormone sensitive lipase (*Hsl*), functions in lipid metabolism, in the fed state *Hsl* does not localise to the lipid droplets, however, during starvation there is an accumulation of *Hsl* on the surface of the lipid droplets (Bi et al., 2012). When *fs(1)h* was knocked down in the fat body, there was no change in the transcript level of *Hsl* between the knockdown in either whole fly (Figure 4-13A) or dissected fat body samples (Figure 4-13A') compared to the control genotype. The *brummer* (*bmm*) lipase plays key roles in both basal and stimulated lipolysis (Kühnlein, 2012). *bmm* knockdown increases lipid storage in *Drosophila* tissue culture, whereas over-expression protects flies against high fat diet-induced TAG accumulation (Grönke et al., 2005). Here, we showed that *fs(1)*h fat body knockdown flies had a decrease in *bmm* transcript levels, but this was not significant in the whole fly (Figure 4-13B). However, there was a significant reduction of *bmm* in the dissected fat body samples following *fs(1)h* knockdown (Figure 4-13B'). The *perilipins* are members of an evolutionarily conserved family of lipid droplet-associated proteins that modulate storage lipid homeostasis (Kimmel and Sztalryd, 2016). *plin1* increases lipolytic activity by recruiting lipases to the lipid surface (Beller et al., 2010) and was significantly reduced in the whole fly samples following *fs(1)h* knockdown in the fat body (Figure 4-13C). *plin1* was further decreased in the dissected fat body samples of *fs(1)h* knockdown flies (Figure 4-13C'). In contrast to the prolipolytic *plin1*, *plin2* protects triglyceride stores in a dose-dependant manner (Tsai et al., 2017). *plin2* transcript levels showed no change in the whole fly samples of *fs(1)h* fat body knockdown flies (Figure 4-13D), however a significant reduction was shown following fat body dissection (Figure 4-13D'). Overall, these data showed a reduction, but not an elimination of *bmm*, *plin1 and plin2* following *fs(1)h* knockdown in the fat body following fat body dissection. These data indicate that the reduction in lipases may be

partially influencing the dysfunction in triglyceride mobilisation following the loss of *fs(1)h* in the fat body.

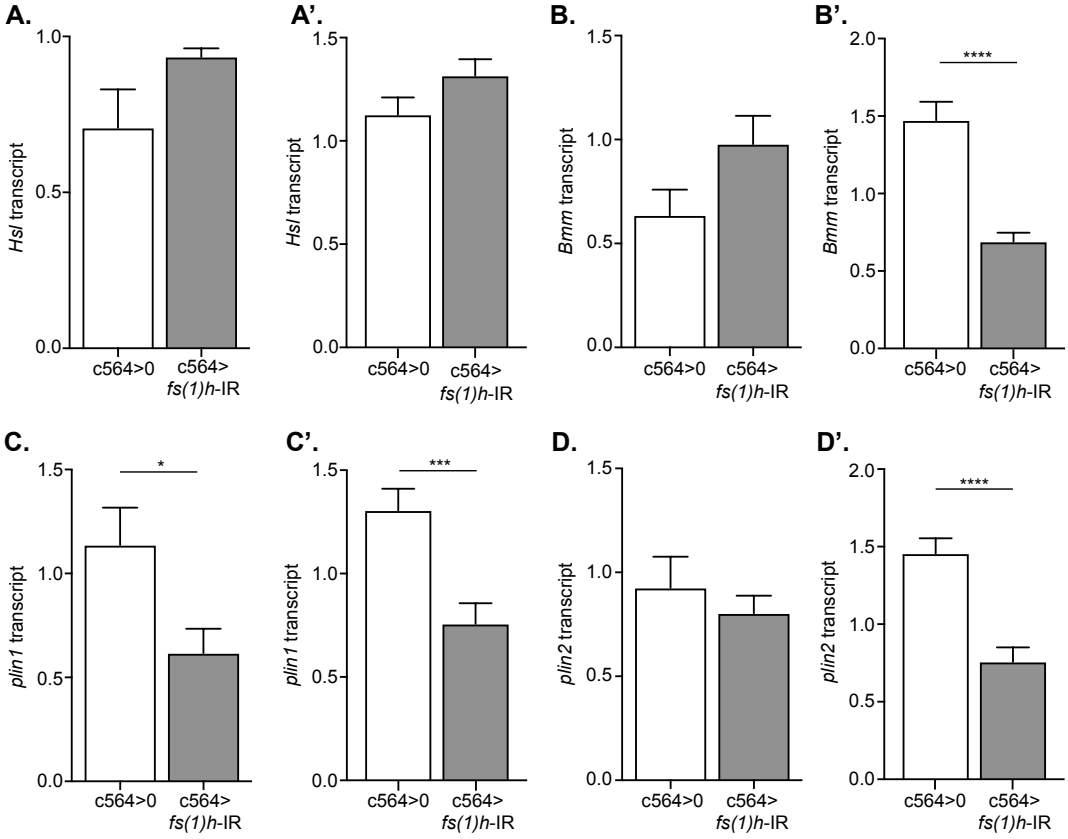


Figure 4-13: Lipid metabolism genes were altered following fat body knockdown of *fs(1)h* in both whole fly samples and dissected fat body

RT-qPCR analysis of 5-7 day old whole flies and dissected fat body following knockdown of *fs(1)h* in the fat body (*c564>fs(1)h*-IR, grey bars) and control samples (*c564*>0, white bars). All RT-qPCR data was normalised to the housekeeping gene, α*-tubulin*. Normalised transcript levels of **A.** *Hormone sensitive lipase* (*Hsl*) in whole fly samples, **A.'** *Hsl* in dissected fat body samples, **B.** *brummer* (*bmm*) in whole fly samples, **B.'** *bmm* in dissected fat body samples, **C.** *perilipin 1* (*plin1*) in whole fly samples, **C.'** *plin1* in dissected fat body samples, **D.** *perilipin 2* (*plin2*) in whole fly samples and **D.'** *plin2* in dissected fat body samples. Unpaired t tests, *p<0.05, ***p=0.001, ****p<0.0001, no stars indicate lack of statistical significance. Data represented as mean of n=8/10 biological repeats per genotype (3 flies per sample), error bars as SEM.

4.8. Flies lacking *fs(1)h* in the fat body were hypoglycemic

In many insects, including *Drosophila,* trehalose, a non-reducing disaccharide, is the main circulating hemolymph sugar, along with low levels of glucose also found in the hemolymph (Reyes-DelaTorre et al., 2012). Trehalose is produced from glucose, and due to its chemical properties has the advantage of protecting *Drosophila* against environmental stresses such as starvation (Matsuda et al., 2015). Glycogen is synthesised from glucose, derived mainly from dietary carbohydrates and amino acids, it comprises the major form of energy storage for carbohydrates and is stored in the *Drosophila* fat body (Arrese and Soulages, 2010). Additionally, when trehalose reaches a certain concentration within the fat body, its production is repressed and the glucose present is used for glycogen synthesis (Friedman, 1978). Interestingly, with increasing age *Drosophila* are shown to have a decrease in trehalose and glycogen, whereas glucose remains constant (Morris et al., 2012). *Drosophila* insulin-like peptides (DILPs) maintain hemolymph sugar levels and the mobilisation of hemolymph trehalose to glucose is critical for metabolic homeostasis. However, the physiological importance of circulating sugar metabolism following changes in nutritional state remain unclear (Yasugi et al., 2017). Flies with defects in energy mobilisation from the fat body, are often found to have altered levels of circulating sugars and stored carbohydrates (Baker and Thummel, 2007). Flies with *fs(1)h* knocked down in the fat body were hypoglycemic compared to the control group, they had reduced circulating sugars, in the form of trehalose and glucose (Figure 4-14A, B). The loss of *fs(1)h* in the fat body also induced a reduction in the stored carbohydrates, glycogen, compared to the control group (Figure 4-14C). Interestingly, we found decreased levels of circulating and stored carbohydrates in *fs(1)h* knockdown flies. This phenotype is reminiscent of those seen in mice heterozygous for *Brd2* mutation, which exhibit reduced serum glucose levels (Wang et al., 2009).

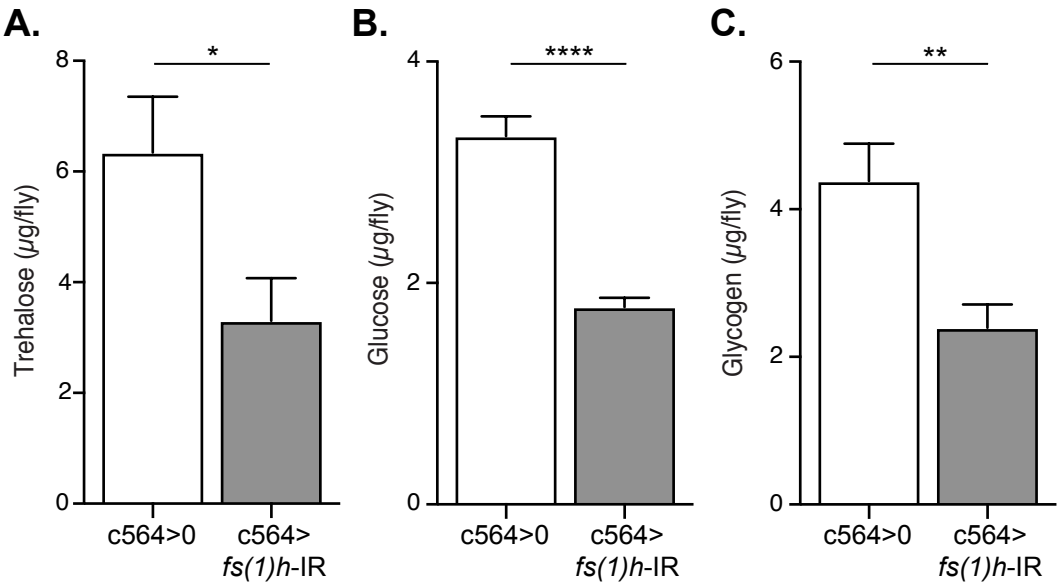


Figure 4-14: *fs(1)h* knockdown in the fat body caused hypoglycemia
Trehalose, glucose and glycogen content in 5-7 day old male flies following knockdown of *fs(1)h* in the fat body (*c564>fs(1)h*-IR, grey bars) and control samples (*c564*>0, white bars). Circulating sugars of **A.** Trehalose*,* **B.** Glucose*,* and stored carbohydrate levels of **C.** Glycogen*.* Unpaired t tests, *p<0.05, **p<0.01, ****p<0.0001. Data represented as mean of n=6 biological repeats per genotype (3 flies per sample), error bars as SEM.

4.8.1. Glucose uptake was not impaired when *fs(1)h* was knocked down in the fat body

In human diseases such as diabetes, there is a failure to increase glucose uptake into peripheral tissues in response to insulin, leading to chronically increased levels of glucose in circulation, which is known as hyperglycemia (Alfa and Kim, 2016). Although the *fs(1)h* knockdown flies were hypoglycemic, we wanted to assay the ability of these flies to take up glucose into their tissues. The fluorescently labelled deoxyglucose analog, 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-Deoxyglucose (2-NBDG), was used to monitor this glucose uptake. These data showed that there was no obvious difference in the way the glucose analog was being taken up by either the *fs(1)h* knockdown or the control group (Figure 4-15). One-hour post injection, we found an organism-wide distribution and uptake of 2-NBDG into the tissues, with no preference for specific tissues. We concluded from these results that glucose uptake and distribution did not seem to be disturbed following the loss of *fs(1)h* in the fat body. It would also be of interest to include a negative control within this experiment, perhaps insulin receptor (InR) mutant flies to show their inability to take-up the 2-NBDG efficiently into the tissues.

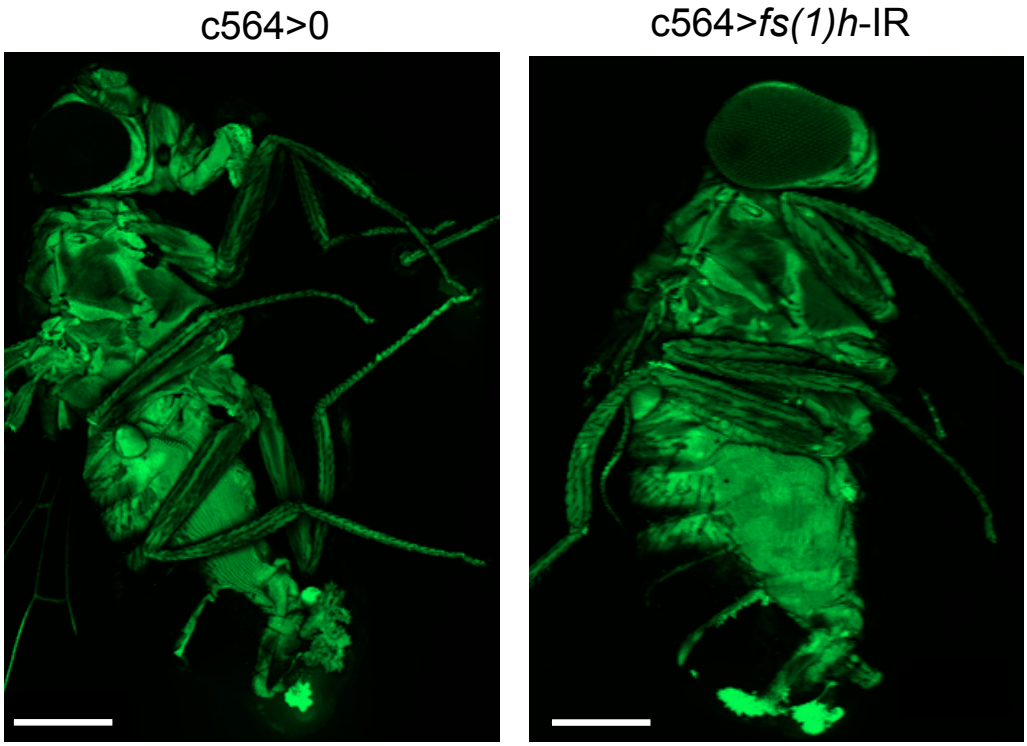


Figure 4-15: Flies with a loss of *fs(1)h* in the fat body were able to take up glucose normally
Images of 5-7 day old males with knockdown of *fs(1)h* in the fat body (*c564>fs(1)h*-IR, right) or control flies (*c564*>0, left). 1-hour post injection with the fluorescently labelled deoxyglucose, 2-NBDG. Scale bar, 300μm.

4.9. Loss of *fs(1)h* in the fat body reduced the activation of the kinase AKT

The insulin-signalling pathway has conserved roles in regulating growth, metabolism and lifespan and can also affect AMP expression (Becker et al., 2010; Taguchi and White, 2008). Based on our phenotypic observations thus far, we further aimed to assay insulin activity in *fs(1)h* fat body knockdown flies. AKT is the key effector kinase of the insulin-signalling pathway, it is activated at Serine 505 by the activation loop at Threonine 308 by phosphoinositide-dependent kinase 1 (PDK1) and the phosphorylation within the carboxy terminus at Serine 473 (Alessi et al., 1996; Scanga et al., 2000). Analysis showed knockdown of *fs(1)h* in the *Drosophila* fat body led to a reduction in phosphorylated AKT (pAKT) relative to the control group (Figure 4-16A). Loss of AKT activity in the *fs(1)h* knockdown flies under normal, fed conditions was indicative of a reduced signalling via the insulin pathway. We further aimed to analyse the levels of p70 S6 kinase (S6K) when phosphorylated at threonine 398, which acts downstream of TOR signalling in response to growth factors and nutrients, was also reduced following knockdown of *fs(1)h* when compared to controls (Figure 4-16B). Interestingly, *Drosophila* deficient in S6K exhibit extreme delays in development and a reduction in body size (Montagne et al., 1999), however this was not observed following *fs(1)h* knockdown in the fat body. As mentioned previously, a reduction in pAKT is often induced by a reduction in insulin signalling and a loss of insulin signalling in a tissue can result in insulin resistance (Rask-Madsen and Kahn, 2012). We tested if the loss of *fs(1)h* in the fat body resulted in insulin resistance by challenging *fs(1)h* knockdown flies and controls with either PBS or two different doses of human insulin (low and high). However, when the *fs(1)h* fat body knockdown flies were injected with insulin they did not show signs of insulin resistance and were able to response in the same manner as the control flies (Figure 4-16C). From these results, we could show that the loss of *fs(1)h* in the fat body led to decreased insulin signalling activity, shown by the decrease in activity of the kinase AKT and a reduction in p70 S6 Kinase (S6K) activity. However, *fs(1)h* deficiency in the fat body did not lead to the development of insulin insensitivity.

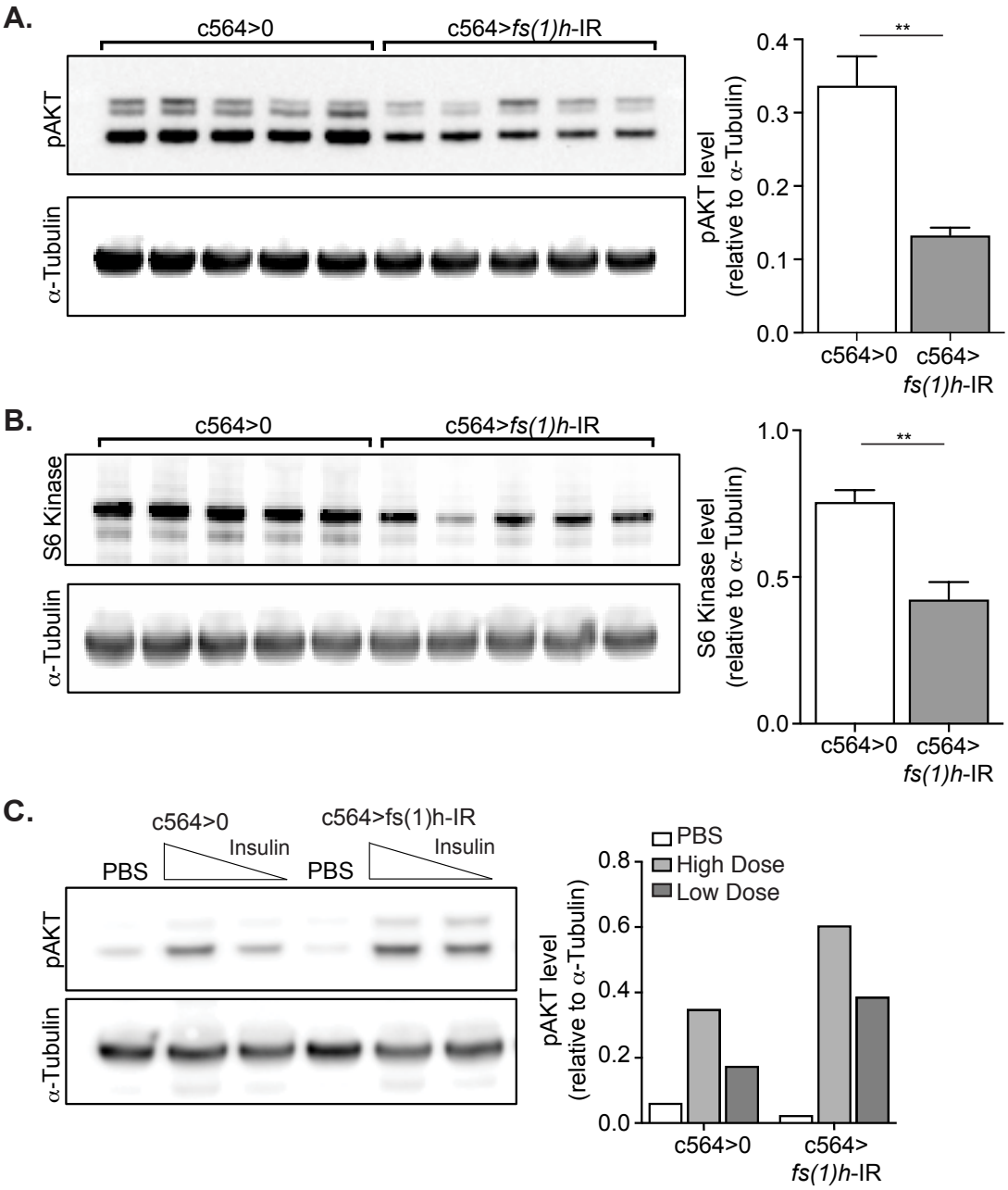


Figure 4-16: *fs(1)h* knockdown flies showed a reduction in pAKT and S6 Kinase but were not insulin resistant

Western blot analysis of 5-7 day old flies following knockdown of *fs(1)h* in the fat body (*c564>fs(1)h*-IR) and controls (*c564*>0). **A.** AKT Ser505 phosphorylation in control (white bars) and *fs(1)h* fat body knockdown (grey bars) flies. Values represented as intensity relative to *α-tubulin*, data shown as mean + SEM, **p<0.01. **B.** p70 S6 Kinase (S6K) in control (white bars) and *fs(1)h* fat body knockdown (grey bars) flies. Values represented as intensity relative to *α-tubulin*, data shown as mean + SEM, **p<0.01. **C.** To assay insulin sensitivity, AKT phosphorylation levels were measured following injection with PBS (white bars) or a high (320pg/fly; light grey bars) or low (64 pg/fly; dark grey bars) dose of human insulin. Values relative to *α-tubulin*.

4.9.1. Insulin signalling related genes showed transcriptional changes following *fs(1)h* knockdown in the fat body

There are a number of key genes in the insulin-signalling pathway and their dysregulation can be detrimental in both vertebrates and invertebrates (Erion and Sehgal, 2013). Several of these key genes are transcriptionally regulated by the phosphorylation event of AKT (Liao and Hung, 2010). Here, we wanted to investigate the expression levels of several key genes of the insulin pathway to determine whether the loss of *fs(1)h* was affecting their transcript levels. The insulin-like receptor (*InR*) is essential for normal development in *Drosophila* and is required for the formation of the epidermis as well as the central and peripheral nervous systems during embryogenesis (Fernandez et al., 1995). Following ligand binding, the InR can activate the insulin-signalling pathway. When *fs(1)h* was knockdown specifically in the fat body, expression level of the *InR* were significantly increased in both the whole fly (Figure 4-17A) and in dissected fat body (Figure 4-17A'). The best-characterised transcriptional effector downstream of AKT is the transcription factor *foxo*. FOXO is phosphorylated by AKT, promoting its nuclear exclusion and limiting gene transcription. In the whole fly, we found no change in transcription levels between the control flies and the *fs(1)h* fat body knockdown flies (Figure 4-17B). However, the transcript level of *foxo* in dissected fat body samples was significantly reduced when compared to controls (Figure 4-17B'). Fitting with this observation, when FOXO levels are high, it is able to reduce its own expression to stimulate the expression of its target genes. *4E-BP*, also known as *Thor*, which is transcriptionally regulated by *foxo* was significantly upregulated in the flies with the *fs(1)h* knockdown in the fat body in both whole fly (Figure 4-17C) and dissected fat body (Figure 4-17C'). Here, we found that loss of *fs(1)h* in the fat body resulted in transcriptional dysregulation of several key genes involved in the insulin pathway. In particular, we found elevated levels of *InR* and *4E-BP*, which are both *FOXO* target genes. These data, along with the reduction in activated AKT shown previously suggest hyperactivation of FOXO.

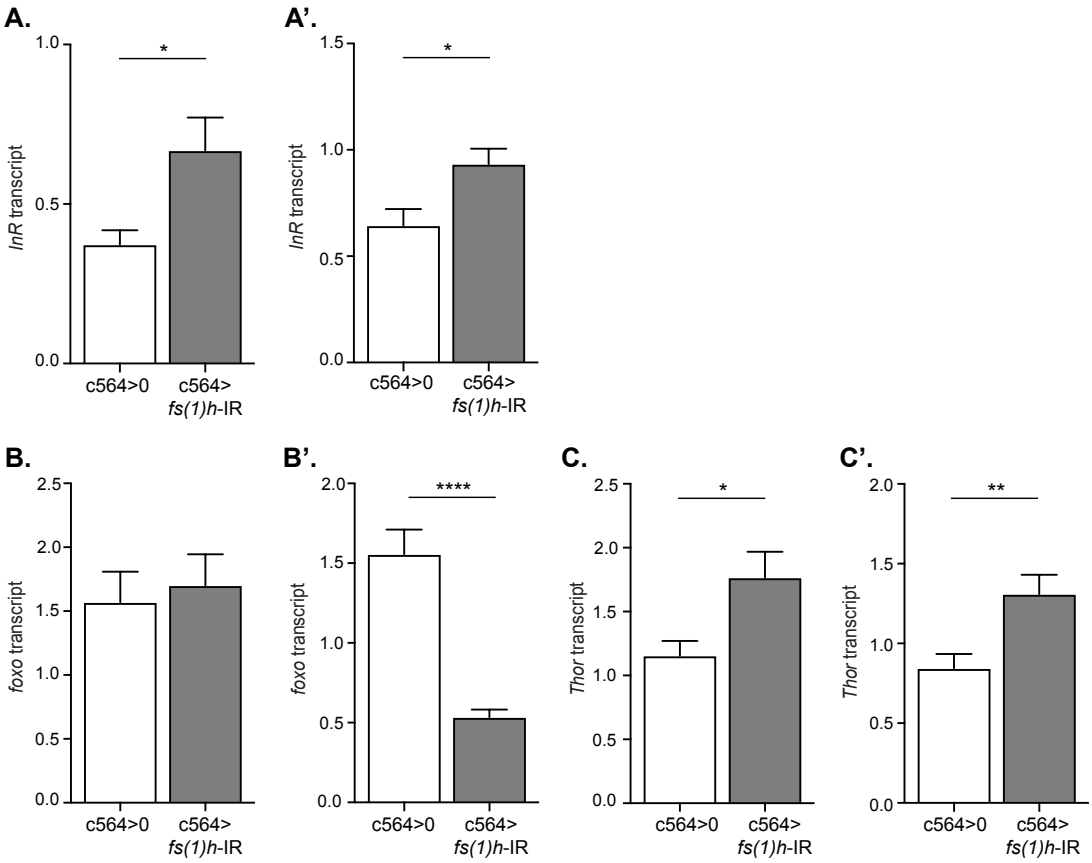


Figure 4-17: Transcript levels of insulin signalling genes were altered following *fs(1)h* knockdown in the fat body

RT-qPCR analysis of 5-7 day old whole flies and dissected fat body samples following knockdown of *fs(1)h* in the fat body (*c564>fs(1)h*-IR, grey bars) and control samples (*c564>*0, white bars). All RT-qPCR data was normalised to the housekeeping gene, α*-tubulin*. Normalised transcript levels of **A.** *Insulin-like receptor* (*InR*) in whole fly samples, **A.'** *InR* in dissected fat body samples, **B.** *foxo* in whole fly samples, **B.'** *foxo* in dissected fat body samples, **C.** *4E-BP* (*Thor*) in whole fly samples and **C.'** *4E-BP* in dissected fat body samples**.** Unpaired t tests, *p<0.05, **p=0.01, ****p<0.0001, no stars indicate lack of statistical significance. Data represented as mean of n=8/10 biological repeats per genotype (3 flies per sample), error bars as SEM.

4.9.2. *Drosophila* insulin-like peptides were increased when *fs(1)h* was knocked down in the fat body

As in mammals, sugar levels in *Drosophila* are regulated by the action of two endocrine hormones, the *Drosophila* insulin-like peptides (DILPs) and the glucagon-like peptide, Akh (Yasugi et al., 2017). In most cases, DILPs signal via the conserved *InR-PI3K* pathway and the best-known transcriptional effect involves the signal-responsive inactivation of FOXO (Britton et al., 2002). DILP2, 3 and 5 are believed to play roles analogous to that played by mammalian insulin in regulating physiology in response to changes in nutritional state (Zhang et al., 2009). DILP secretion is required for the activation of insulin signalling and to regulate glucose levels within tissues and in the hemolymph. Due to the changes in activated AKT levels, as well as the changes in carbohydrate levels and changes in the transcript level of various insulin signalling genes we observed after the loss of *fs(1)h* in the fat body, we assayed DILP levels following *fs(1)h* knockdown in the fat body. The insulin-like peptides, *DILP2*, *3* and *5*, released from IPCs in the brain were all shown to be significantly upregulated following *fs(1)h* knockdown (Figure 4-18A). *DILP6*, which is released from the fat body itself, was measured in whole fly and dissected fat body following *fs(1)h* knockdown, although there was very little transcriptional change in either sets of samples (Figure 4-18B, B'). Additionally, the transcript level of phosphoenolpyruvate carboxykinase (*Pepck*), a key enzyme in gluconeogenesis (Quinn and Yeagley, 2005), was significantly upregulated in whole fly samples following *fs(1)h* knockdown in the fat body (Figure 4-18C). However, this observation was lost in the dissected fat body samples and the transcript levels were similar in both the control group and following *fs(1)h* knockdown (Figure 4-18C'). We conclude that even though there was a reduction in insulin signalling activity following *fs(1)h* knockdown in the fat body, we found elevated levels of *DILP2*, *3* and *5* in these flies which may be an attempted rescue mechanism induced by the fat body to increase AKT activation and reduced FOXO levels.

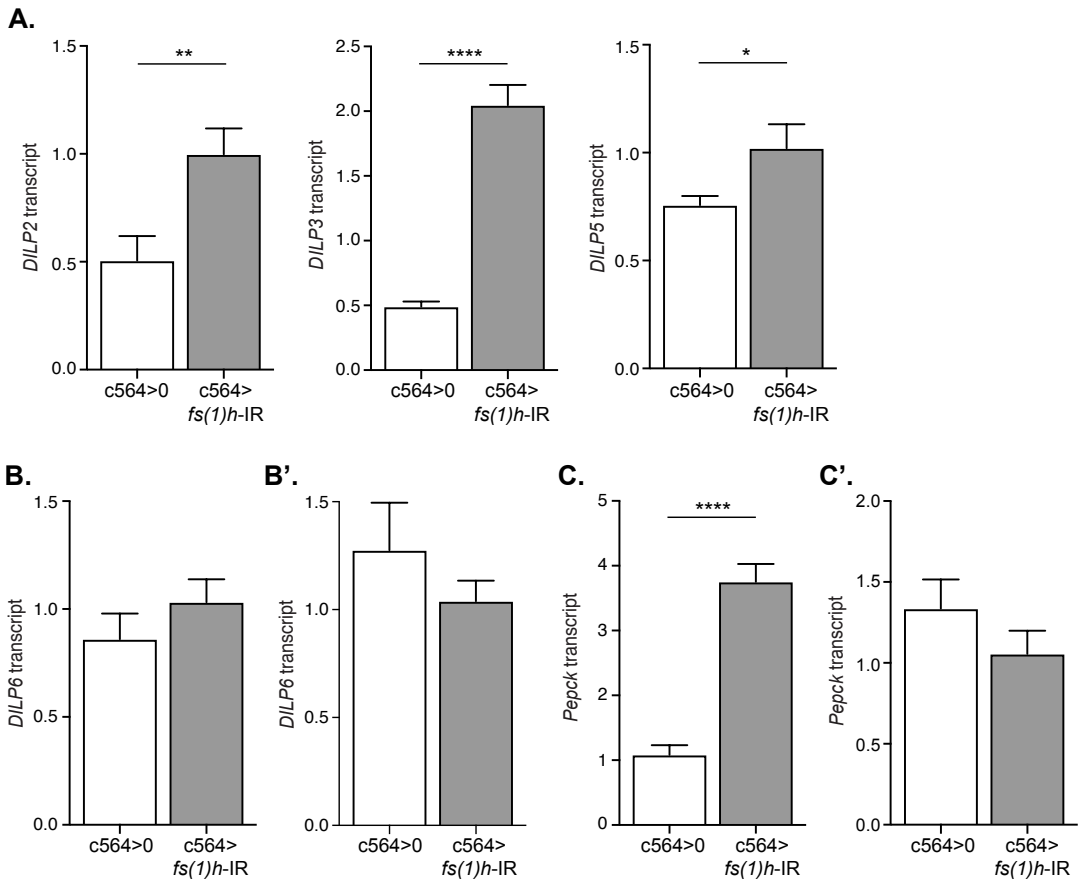


Figure 4-18: DILP expression was increased following knockdown of *fs(1)h* in the fat body

RT-qPCR analysis of 5-7 day old whole fly samples and dissected fat body following knockdown of *fs(1)h* in the fat body (*c564>fs(1)h*-IR, grey bars) and control samples (*c564*>0, white bars). All RT-qPCR data was normalised to the housekeeping gene, α*-tubulin*. Normalised transcript levels of **A.** *Drosophila insulin-like peptide 2, 3 and 5* (*DILP2, 3* and *5*) in whole fly samples, **B.** *DILP6* in whole fly samples, **B'.** *DILP6* in dissected fat body samples, **C.** *Phosphoenolpyruvate carboxykinase* (*Pepck*) in whole fly samples and **C'.** *Pepck* in dissected fat body samples**.** Unpaired t tests, *p<0.05, **p<0.01, ****p<0.0001, no stars indicate lack of statistical significance. Data represented as mean of n=8/10 biological repeats per genotype (3 flies per sample), error bars as SEM.

4.10. Fat body-derived nutrient-responsive signals were altered when *fs(1)h* was removed from the fat body

The fat body plays a central role in the regulation of systemic growth in response to nutrients. Nutrient sensors function as detectors of nutrient flux via metabolic pathways within cells and in the hemolymph (Miyamoto et al., 2013). The fat body senses nutrients via two main mechanisms, firstly by sensing intracellular amino acids via TOR signalling (Colombani et al., 2003) or by releasing or initiating the release of DILPs from the fat body (DILP6) or from other tissues such as the IPCs in the brain (DILP2, 3 and 5) (Owusu-Ansah and Perrimon, 2014).

upd1 and *upd2,* two ligands of the Jak/STAT pathway, both play important roles in nutrient sensing (Beshel et al., 2017; Rajan and Perrimon, 2012). *upd1*, considered to be a mammalian leptin homolog, is expressed in the *Drosophila* brain and plays an important role in food intake and food cue attraction (Beshel et al., 2017). Interestingly, transcript levels of *upd1* were significantly increased in the flies lacking *fs(1)h* in the fat body compared to the control flies (Figure 4-19A). However, this significant increase was not shown in the dissected fat body samples, although there was a similar increasing trend (Figure 4-19A'). *upd2,* also considered to be a functional analogue of leptin, is sugar- and lipid-sensitive, and acts independent of the TOR pathway (Sano et al., 2015). upd2 secretion from the fat body is also associated with the release of DILPs from the IPCs in the brain (Rajan and Perrimon, 2012).The transcript levels of *upd2* following *fs(1)h* knockdown showed an increasing trend in whole fly samples (Figure 4-19B), and following fat body dissection the transcript level of *upd2* was significantly upregulated compared to the control group (Figure 4-19B'). Two other peptides, known as Growth-blocking peptide 1 and 2 (Gbp1, Gbp2) are produced by the *Drosophila* fat body in response to amino acids and TOR signalling and are thought to act as nutrient sensing factors (Koyama and Mirth, 2016). *Gbp1* and *2* also, either directly or indirectly, stimulate DILP secretion from IPCs, to increase insulin-like growth factor signalling activity (Koyama and Mirth, 2016). *Gbp1* regulates the immune response and its expression is sensitive to starvation (Koyama and Mirth, 2016). When we assayed the transcript levels of *Gbp1*, they remained unchanged in whole fly samples following *fs(1)h* knockdown (Figure 4-19C). Conversely, following fat body dissection there was a significant reduction in *Gbp1* levels when *fs(1)h* was removed in the fat body (Figure 4-19C'). Levels of *Gbp2* were also assayed and were significantly reduced following *fs(1)h* knockdown in the whole fly and in the dissected fat body tissue (Figure 4-19D and D'). *CChamide-2* (*CCHa2*), much like *upd2*, is sugar- and lipid-sensitive humoral signal secreted by the fat body to regulate DILP

secretion (Sano, 2015). *fs(1)h* knockdown in the fat body led to a significant reduction in *CCHa2* transcript levels following knockdown compared to controls in both the whole fly samples and the dissected fat body tissue (Figure 4-19E and E'). Another regulator of nutrient sensing is *ImpL2*, the secreted insulin/IGF antagonist is involved in the wasting process by decreasing systemic insulin signalling (Kwon et al., 2015). *ImpL2* was significantly upregulated in whole fly samples following *fs(1)h* knockdown (Figure 4-19F). However, in contrast, when the fat body was dissected there was a significant down-regulation of *ImpL2* following *fs(1)h* knockdown in the fat body (Figure 4-19F'). Our findings showed that various nutrient sensing factors were dysregulated following *fs(1)h* knockdown in the fat body, including a significant increase in the negative regulator of DILPS, *ImpL2* and the leptin analogue *upd2.* However, it is unknown whether these changes were primarily caused by an insufficiency of *fs(1)h* in the fat body, or were secondary effects due to the severe changes in the insulin-signalling pathway found in *fs(1)h* fat body knockdown flies.

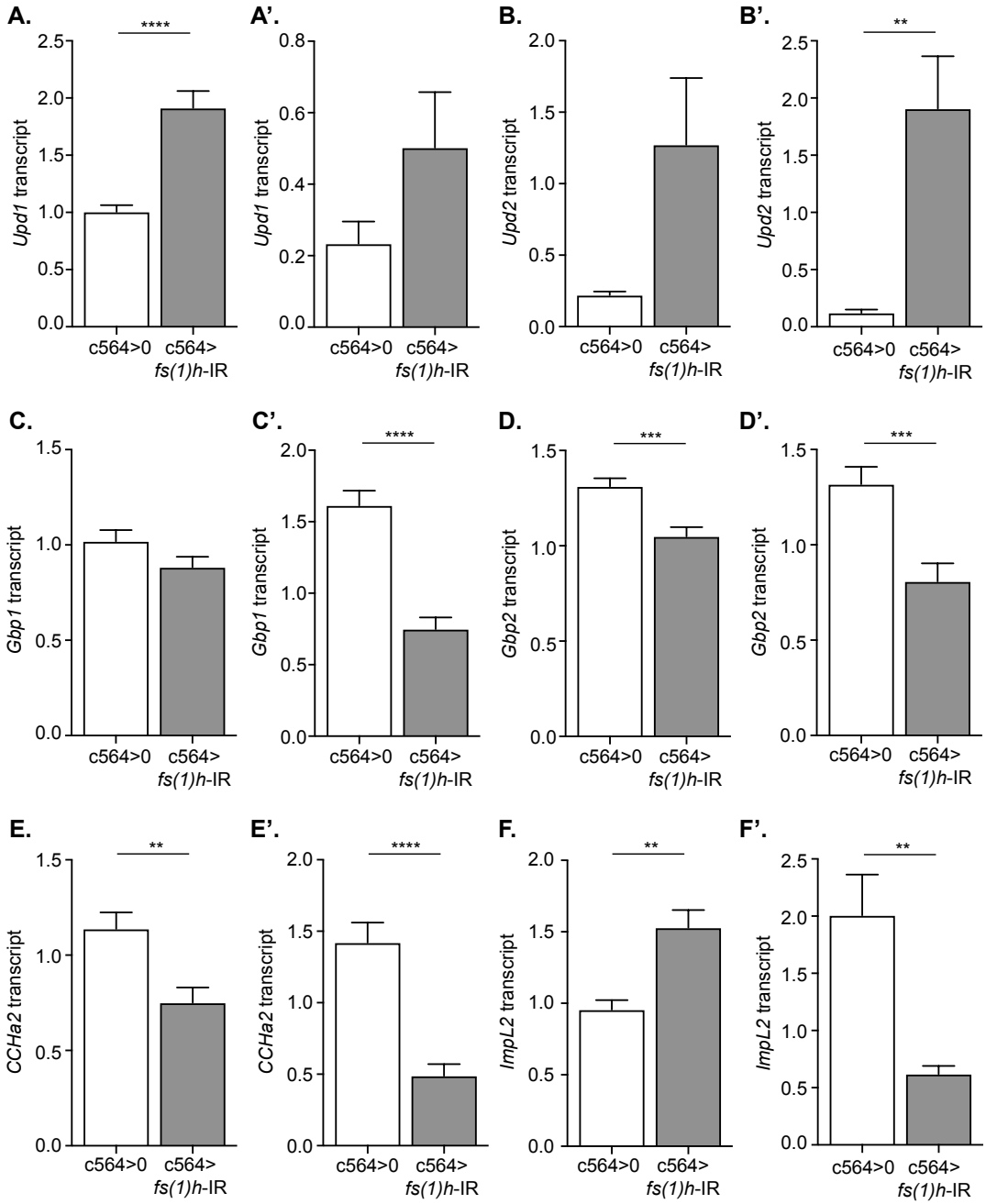


Figure 4-19: Transcript levels of insulin regulatory signals varied following the loss of *fs(1)h* in the fat body

RT-qPCR analysis of 5-7 day old whole flies and dissected fat body following knockdown of *fs(1)h* in the fat body (*c564>fs(1)h*-IR, grey bars) and control samples (*c564*>0, white bars). All RT-qPCR data was normalised to the housekeeping gene, α*-tubulin*. Normalised transcript levels of **A.** *unpaired 1* (*upd1*) in whole fly samples, **A.'** *upd1* in dissected fat body samples, **B.** *unpaired 2* (*upd2*) in whole fly samples, **B.'** *upd2* in dissected fat body samples, **C.** *Growth-blocking peptide 1* (*Gbp1*) in whole fly samples, **C'.** *Gbp1* in dissected fat body samples, **D.** *Growth-blocking peptide 2* (*Gbp2*) in whole fly samples, **D'.** *Gbp2* in dissected fat body samples, **E.** *CCHamide-2* (CCHa2) in whole fly samples, **E'.** *CCHa2* in dissected fat body samples, **F.** *Ecdysone-inducible gene L2* (*ImpL2*) in whole fly samples and **F'.** *ImpL2* in dissected fat body samples**.** Unpaired t tests, **p=0.01, ***p=0.001, ****p<0.0001, no stars indicate lack of statistical significance. Data represented as mean of n=8/10 biological repeats per genotype (3 flies per sample), error bars as SEM.

4.11. The phenotypes observed were reproduced with a second, non-overlapping *fs(1)h* RNAi line

To verify the specificity of the *fs(1)h* knockdown achieved using the original *fs(1)h*-IR line (KK108662), we wanted to demonstrate the finding with a second non-overlapping *fs(1)h* RNAi line. Using a second *fs(1)h* RNAi line, GD51772 (*fs(1)h*-IR II), we showed a similar reduction in survival compared to controls at both 25°C and 29°C (Figure 4-20A). Following infection with *L. monocytogenes* or *F. novicida,* again we saw a similar reduced lifespan compared to the control flies (Figure 4-20B). Furthermore, the second *fs(1)h* RNAi line showed increased antimicrobial peptide transcript levels (Figure 4-20C), and finally it showed a significant reduction in activated AKT following knockdown in the fat body (Figure 4-20D). Therefore, we can confirm the effects and phenotypes described with the originally RNAi line (KK108662) are specific to the loss of the target gene, *fs(1)h*.

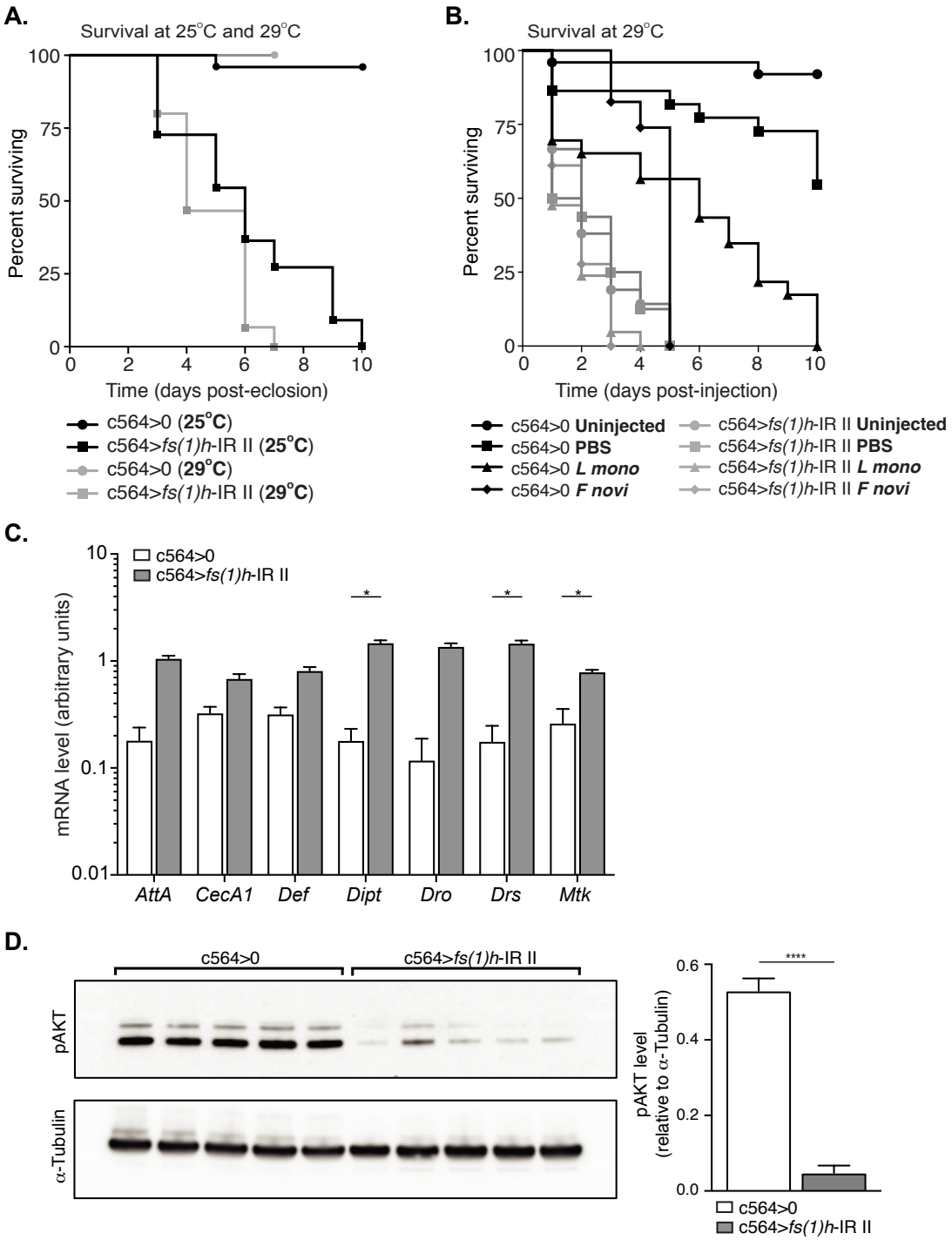


Figure 4-20: An alternative *fs(1)h* RNAi line replicated the data from the original RNAi line

Experiments carried out with another *fs(1)h* RNAi line, GD51227 at 5-7 days old. **A.** Survival was carried out comparing newly eclosed *fs(1)h* fat body knockdown flies (*c564>fs(1)h*-IR II, squares) and control samples (*c564*>0,circles) at 25°C (black lines) and 29°C (grey lines). n=20 per genotype. **B.** Survival was carried out on 5-7 day old male flies following knockdown of *fs(1)h* in the fat body (*c564>fs(1)h*-IR II, grey lines) and control samples (*c564*>0, black lines) and bacterial infection. Uninjected flies (circles), PBS injected (squares), *Listeria monocytogenes* infected (triangles, OD 0.1) or *Francisella novicida* infected (diamonds, OD 0.1). At 29°C, n=20 per genotype. **C.** RT-qPCR analysis of whole fly samples following knockdown of *fs(1)h* in the fat body (*c564>fs(1)h*-IR II, grey bars) and control samples (*c564*>0, white bars). All RT-qPCR data was normalised to the housekeeping gene, *α-tubulin*.

Data shown on log10 scale. Normalised transcript levels of *Attacin, Cecropin A1, Defensin, Diptericin, Drosocin, Drosomycin* and *Metchnikowin.* Unpaired t tests, *p<0.05, no stars indicate lack of statistical significance. Data represented as mean of n=6 biological repeats per genotype (3 flies per sample), error bars as SEM. **D.** AKT Ser505 phosphorylation in control (*c564*>0; white bars) and *fs(1)h* fat body knockdown (*c564>fs(1)h*-IR II, grey bars) flies. Values represented as intensity relative to *α-tubulin*, data shown as mean + SEM, ****p<0.0001.

4.12. Chapter 4 overview

In this chapter, we identified that the bromodomain-containing protein, *female sterile (1) homeotic* (*fs(1)h),* the sole member of the BET family, is required in the fat body of *Drosophila* for normal immune function and metabolism.

fs(1)h is known to be important in *Drosophila* body planning and development (Florence and Faller, 2008), however, its role in the immune response and metabolism had not been investigated until now. We have shown in Chapter 4 that the loss of *fs(1)h* in the fat body caused a number of immune and metabolic phenotypes. A number of these phenotypes were also confirmed using either a second, non-overlapping *fs(1)h* RNAi (GD51227) and a different fat body driver (*r4-Gal4*). Survival assays showed that fat body knockdown of *fs(1)h* led to a drastic reduction in survival following infection, but also under physiological, uninfected conditions. Further analysis of these flies also showed an increase in antimicrobial peptide (AMP) expression when uninfected or infected with bacteria. The transcription factor *Relish*, an important *NF-κB* family member of the *Imd* pathway was upregulated at total protein levels, however, the cleaved C-terminal fragment that remains in the cytoplasm after Relish activation was not upregulated following *fs(1)h* knockdown in the fat body. Additionally, following the loss of *fs(1)h* in the fat body, there was an upregulation of inflammatory cytokines, which play important roles in controlling immune response. Knocking down *fs(1)h* in the fat body also resulted in a number of metabolic phenotypes. When assaying triglyceride levels in the *fs(1)h* knockdown flies, we found no change in the quantity or the morphology of the triglycerides stored in fat body cells. However, the flies had an inability to utilise their triglyceride stores following 24-hour starvation and a slight reduction in gut integrity when *fs(1)h* was removed from the fat body, but they showed no changes in endogenous bacterial load. Furthermore, *fs(1)h* knockdown flies showed a reduction in a number of important lipases, including *bmm, plin1* and *plin2,* which are all involved in lipid mobilisation, fitting with the inability for the flies to utilise their triglyceride stores effectively. When both circulating and stored sugars were measured it was shown these flies were hypoglycemic when *fs(1)h* was knocked down in the fat body, but glucose uptake into the tissues was not impaired or dysregulated by the fat body knockdown. Removing *fs(1)h* in the fat body also led to a reduction in the levels of

activated AKT and S6 kinase, though it did not lead to the development of insulin resistance as these flies were able to respond as normal to injections of insulin. Along with the change in activated AKT, we also saw dysregulation in a number of insulin pathway genes, including *DILP*s, which are released from ICPs in the brain to activate the insulin-signalling pathway. Interestingly, there was also an increased expression of the *foxo* target genes, *InR* and *4E-BP*. A number of known fat body-derived insulin-regulatory and nutrient-sensing signals including *Gbp1, Gbp2 and CCHa2* were strongly reduced, and *upd2* was strongly upregulated following knockdown of *fs(1)h* in the fat body.

These data together showed the effects of *fs(1)h* knockdown in the fat body are complex, including both immune and metabolic phenotypes. So far it is unclear whether the pathologies observed were due to cell-autonomous effects on insulin signalling, or if it was mediated in part by dysregulated endocrine signals, which resulted collectively in impairment of normal physiological insulin signalling. The best-characterised transcriptional effector downstream of AKT in the insulin pathway is FOXO. Under normal physiological conditions, AKT phosphorylates FOXO, rendering it unable to move into the nucleus to initiate the expression of an array of transcription factors. Due to the roles of *foxo*, our observation of *fs(1)h* knockdown increasing AMP expression and the reducing in AKT activity could suggest there may be an increase in FOXO activity. The next chapter in this thesis will further explore the role of FOXO when *fs(1)h* is knocked down in the fat body.

Chapter 5

The role of *foxo* in *Drosophila* with *fs(1)h* knocked down in the fat body

5.1. Introduction

foxo transcription factors are part of a large family of Forkhead proteins; they are transcriptional regulators that are characterised by a conserved DNA-binding domain (Greer and Brunet, 2005). In humans, there are 39 members of the forkhead family that have an array of functions. Within the human forkhead family, the FOXO subgroup contains four main groups, FOXO1, FOXO3a, FOXO4 and FOXO6. *Foxo1* mRNA is abundant in adipose tissue (Chakrabarti and Kandror, 2009), *Foxo3a*, shows high mRNA expression in the brain (Kannike et al., 2014) and *Foxo4* is highly expressed in the heart (Zhu et al., 2015). *Foxo6* is expressed highly in the developing brain, suggesting it may have an important role in central nervous system (CNS) development (Salih et al., 2012). *foxo* has many varying functions in mammalian cells including DNA binding, cell differentiation, cell death, stress resistance and glucose metabolism (Fu and Tindall, 2008; Gross et al., 2008; Martins et al., 2016; Obsil and Obsilova, 2011; Sunayama et al., 2011). FOXO is a direct substrate for the protein kinase AKT in response to stimulation by insulin or growth factors (Greer and Brunet, 2005). The phosphorylation of FOXO by AKT leads to a change in subcellular localisation of FOXO, in the absence of insulin or other growth factors, when insulin signalling is inactive, FOXO can localise to the nucleus, bind to DNA and initiate the transcription of a range of genes. The degradation of FOXO is mediated by the ubiquitin-proteasome pathway and AKT activity is necessary for ubiquitin mediated degradation of FOXO1 and FOXO3a (Aoki et al., 2004; Huang et al., 2005; Matsuzaki et al., 2003; Plas and Thompson, 2003).

In *Drosophila*, there is a single isoform of *foxo*, which is most similar to nematode *daf-16* and *FOXO3a* in mammals. This single *Drosophila* isoform is responsible for all the roles of the four mammalian FOXO subgroup (Spellberg and Marr, 2015). *foxo* is highly conserved in *Drosophila* and it plays important roles in regulating the size of cells and organs and is a crucial mediator of insulin signalling (van der Horst and Burgering, 2007; Jünger et al., 2003). It is also a key regulator in stress resistance, ageing and metabolism. Ageing is slowed when insulin signalling is reduced and expression of wild-type *foxo* is adequate to increase longevity in flies (Giannakou and Partridge, 2007; Hwangbo et al., 2004). In response to cellular stress, such as nutrient deficiency, *foxo* is activated and inhibits growth through the upregulation of target genes such as *4E-BP* (Jünger et al., 2003). *foxo* also provides a mechanism for cross-regulation between *Drosophila* innate immunity and metabolism. AMP activation can be achieved by *foxo* independently of the immunoregulatory pathways, *Toll* and *Imd* (Becker et al., 2010). In uninfected flies, in response to

nuclear *foxo* activity, the expression of AMP genes can be stimulated during times of stress, such as starvation (De Gregorio et al., 2002). Additionally, AMP induction is enhanced when *foxo* is over-expressed. It was further shown that *foxo* can bind to the regulatory region of AMPs including *Drosomycin* inducing its transcription (Becker et al., 2010). Furthermore, independent of pathogen-responsive immunity in *Drosophila*, AMPs can be activated under physiological conditions in response to cell- and tissue-specific energy fluctuations (Becker et al., 2010).

As mentioned previously, the fat body, analogous to adipose tissue and the liver in mammals, is a multi-functioning tissue in both immunity and metabolism (Azeez et al., 2014). The transcription factor *foxo* mediates a range of autonomous and non-autonomous signals in the fat body. In *Drosophila,* the insulin-signalling pathway down-regulates *foxo*, however when AKT activation is reduced and *foxo* levels are increased it has been shown to extend lifespan (Giannakou and Partridge, 2007; Hwangbo et al., 2004). Fat body *foxo* regulates ageing when activated in the pericerebral fat body of adult *Drosophila* and mediates feedback signalling between insulin producing cells (IPCs) and the fat body (Hwangbo et al., 2004). *Drosophila* insulin-like peptides (DILPs) released from the IPCs in the brain can repress *foxo* in the fat body, whilst *foxo* can regulate *DILP* expression from the IPCs. *foxo* is also able to regulate the transcription of *DILP6* specifically in the fat body (Slaidina et al., 2009). Furthermore, in mammals and *Drosophila, foxo* is involved in regulating lipolysis in adipocytes and the fat body by controlling the expression of lipases (Chakrabarti and Kandror, 2009).

5.1.1. Objective and aims

Following the loss of *fs(1)h* in the fat body, we found that flies had a reduction in survival, dysregulation of AMP production along with several metabolic pathologies, including an inability to utilise triglyceride stores and hypoglycemia. We also detected a reduction in AKT activity and an increase in the transcript levels of *foxo* target genes, including *InR* and *4E-BP*. To determine the extent in which FOXO hyperactivation may be responsible for the observed phenotypes of *fs(1)h* fat body knockdown flies, we combined *fs(1)h* knockdown with a heterozygous loss-of-function mutation in *foxo*. The objective of this chapter is to investigate whether increased FOXO activity may drive the phenotypes observed when *fs(1)h* is knocked down in the fat body.

The aims of this chapter are:

1. To identify whether removing a single copy of *foxo* can rescue the reduced lifespan and increased AMPs observed following *fs(1)h* knockdown in the fat body.
2. To further analyse if a reduction in *foxo* levels can also rescue the metabolic defects identified following the knockdown of *fs(1)h* in the fat body.

5.2. Confirming the knockdown of *fs(1)h* and *Gal4* expression in flies lacking one copy of *foxo*

Following the removal of one copy of *foxo*, we wanted to ensure that *fs(1)h* was still being knocked down efficiently in the fat body and that *foxo* was not required for the expression of *Gal4.* In whole fly samples, as before, *fs(1)h* transcript levels were not reduced following knockdown in the fat body specifically (Figure 5-1A, also see 4-1A). However, when one copy of *foxo* was removed from the *fs(1)h* knockdown flies, there was a significant upregulation in *fs(1)h* transcript level (Figure 5-1A). In dissected fat body samples, the loss of *fs(1)h* was observed following fat body knockdown, however there was no change in *fs(1)h* transcript levels in the *foxo* heterozygous flies (Figure 5-1A'). These data, showing an increase in whole fly *fs(1)h* levels following the removal of one copy of *foxo* suggests there may be a feedback mechanism between *fs(1)h* and *foxo.* However, as we were unable to detect reduced *fs(1)h* levels in the dissected fat body of heterozygous *foxo* mutants, we wanted to ensure that the increase in *fs(1)h* was due to the reduction in *foxo* and not due to a reduction in the efficiency of the *Gal4* mediating *fs(1)h* knockdown. Assaying *Gal4* expression in whole fly and dissected fat body showed that expression of *c564-Gal4* was enhanced by *fs(1)h*, but *foxo* heterozygosity alone had no effect on *Gal4* expression (Figure 5-1B and B'). We can summarise that *fs(1)h* transcript levels seem to be influenced by levels of *foxo*, which could further suggest a regulatory feedback mechanism.

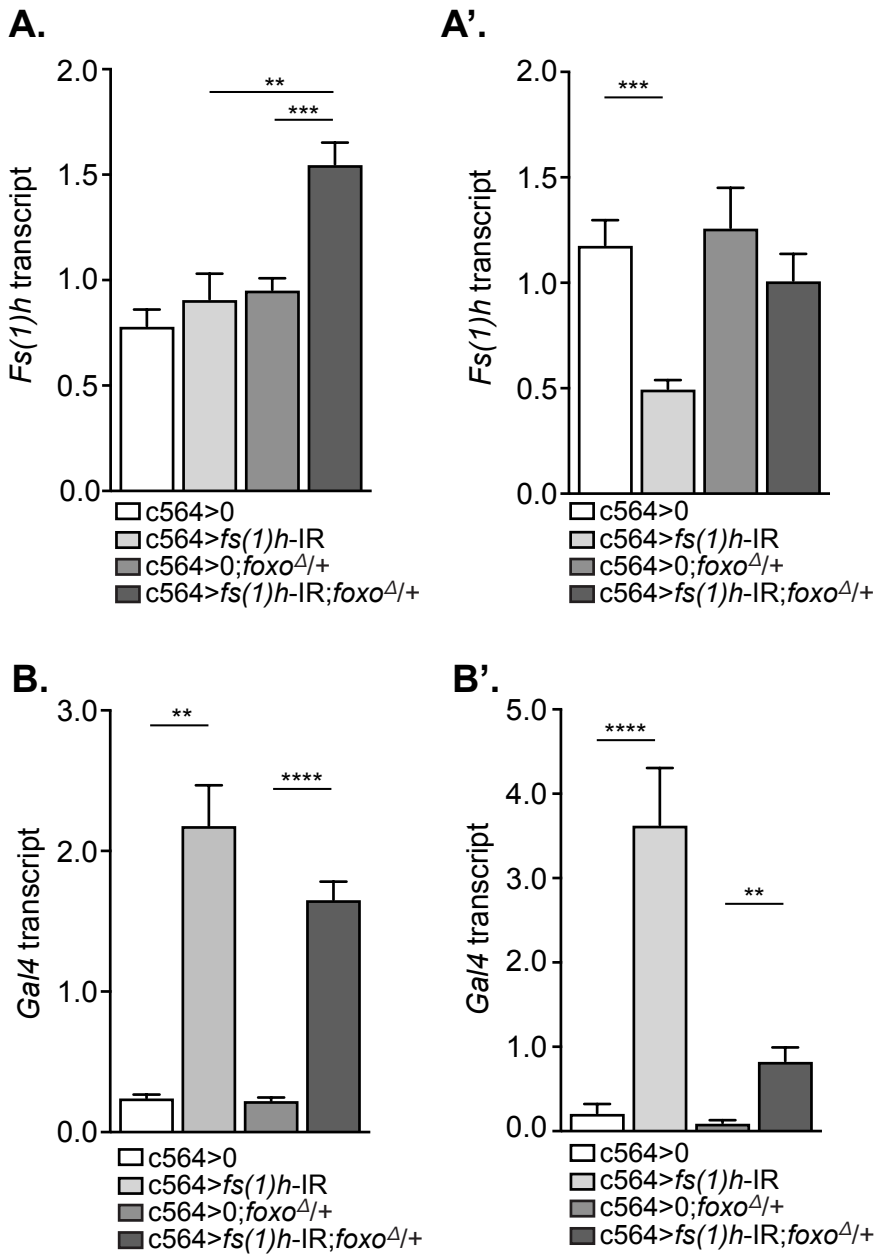


Figure 5-1: *fs(1)h* levels were reduced in dissected fat body samples following knockdown

RT-qPCR analysis of 5-7 day old fly samples following knockdown of *fs(1)h* in the fat body (*c564>fs(1)h*-IR, light grey bars), control samples (*c564*>0, white bars), *fs(1)h* fat body knockdowns heterozygous for *foxo* (*c564>fs(1)h*-IR;*foxo*$^{\Delta}$/+, dark grey bars), and control samples heterozygous for *foxo* (*c564*>0;*foxo*$^{\Delta}$/+, grey bars). All RT-qPCR data was normalised to the housekeeping gene, α*-tubulin*. Normalised transcript levels of **A.** *fs(1)h* expression in whole fly samples with *fs(1)h* knocked down in the fat body, heterozygous for *foxo* and controls. Unpaired t test, data shown as mean + SEM, **p<0.01, ***p<0.001. **A'.** *fs(1)h* expression in dissected fat body fly samples with *fs(1)h* knocked down in the fat body, heterozygous for *foxo* and controls. Unpaired t test, data shown as mean + SEM, ***p<0.001. **B.** *Gal4* expression in whole fly samples with *fs(1)h* knocked down in the fat body, heterozygous for *foxo* and controls. Unpaired t test, data shown as mean + SEM, **p<0.01, ****p<0.0001. **B'.** *Gal4* expression in dissected fat body fly samples with *fs(1)h* knocked down in the fat body, heterozygous for *foxo* and controls. Unpaired t test, data shown as mean +

SEM, **p<0.01, ****p<0.0001, n=8 biological repeats per genotype (3 flies per sample), error bars as SEM.

5.3. Removing one copy of *foxo* rescued the reduced *Drosophila* survival after the loss of *fs(1)h* in the fat body

Loss of *fs(1)h* in the *Drosophila* fat body led to a severe reduction in lifespan, under physiological conditions and following infection. In order to determine whether an increase of FOXO was causing the survival phenotype, one copy of *foxo* was removed in flies with *fs(1)h* knockdown in the fat body. Heterozygosity for the *foxo* null allele rescued the reduced lifespan of *fs(1)h* knockdown nearly back to that of the control group (Figure 5-2A). Removing one copy of *foxo* alone had no effect on the lifespan of the control genotype.

Following bacterial infection with *Francisella novicida*, removing *fs(1)h* in the fat body and one copy of *foxo* did not rescue the survival effect and the flies proceeded to die by day 4 post-infection, the same shortened survival as seen in *fs(1)h* knockdown in the fat body alone (Figure 5-2B). Therefore, we can conclude that reducing the level of *foxo* along with the loss of *fs(1)h* in the fat body could rescue the lifespan of the flies under physiological conditions, but was not sufficient to rescue the decreased survival following bacterial infection.

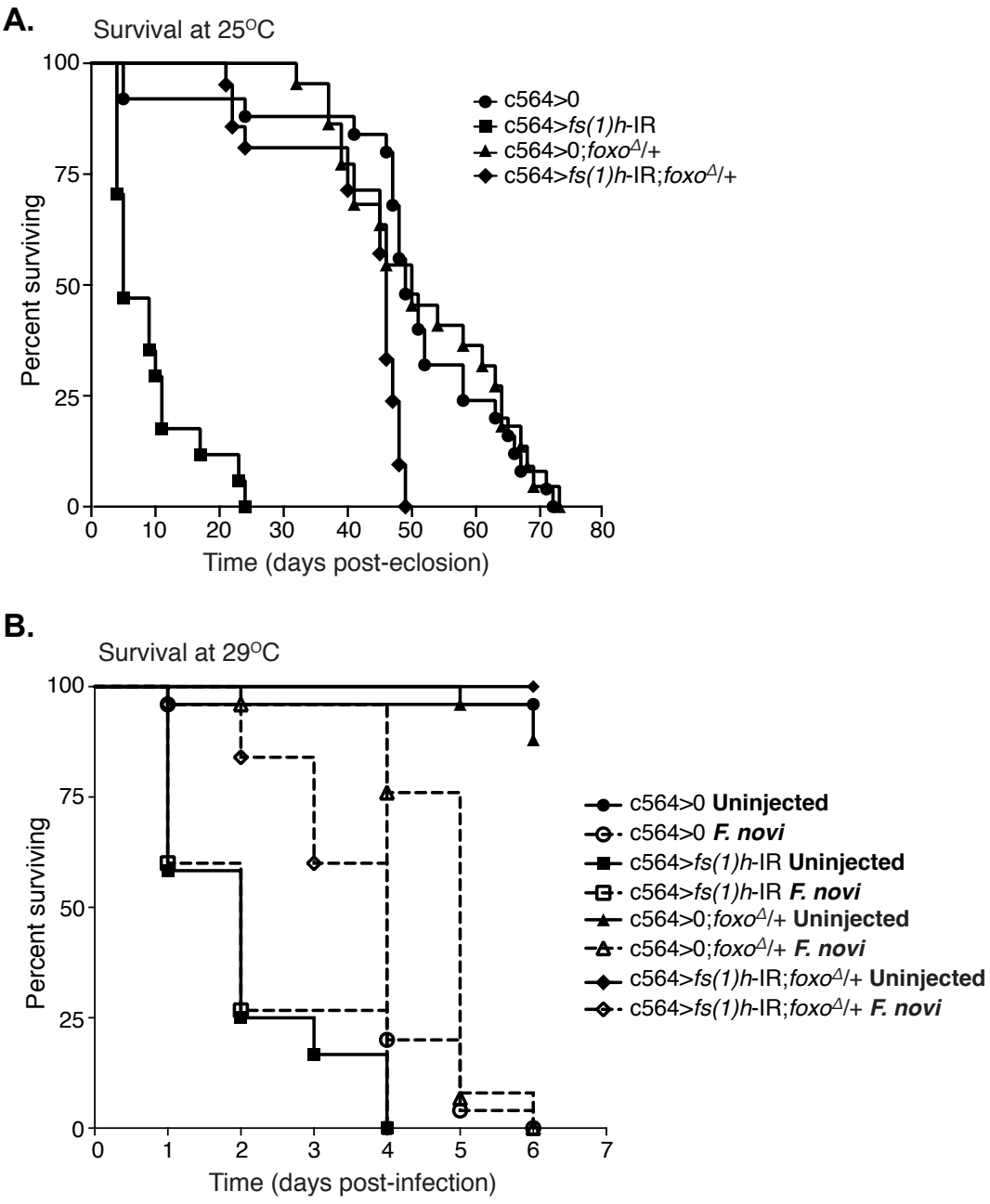


Figure 5-2: Uninfected survival was rescued by removing one copy of *foxo*
Flies were observed daily for the number of deaths and percent survival was calculated over time following knockdown of *fs(1)h* in the fat body, *fs(1)h* knockdown flies heterozygous for *foxo* and controls. **A.** Survival was carried out comparing newly eclosed *fs(1)h* fat body knockdown flies (*c564>fs(1)h*-IR, squares), *fs(1)h* knockdowns also heterozygous for *foxo* (*c564>fs(1)h*-IR;$foxo^{\Delta}$/+, diamonds) and control samples (*c564*>0, circles; *c564*>0;$foxo^{\Delta}$/+, triangles) at 25°C. n=20 per genotype. **B**. Survival was carried out on 5-7 day old male flies following knockdown of *fs(1)h* in the fat body (*c564>fs(1)h*-IR, squares), *fs(1)h* knockdowns also heterozygous for *foxo* (*c564>fs(1)h*-IR;$foxo^{\Delta}$/+, diamonds) and control samples (*c564*>0, circles; *c564*>0;$foxo^{\Delta}$/+, triangles) and bacterial infection with *Francisella novicida* (represented by the dashed lines and open shapes) at 29°C, n=20 per genotype and condition.

5.4. AMP expression levels were normalised following the removal of one copy of *foxo*

Antimicrobial peptides (AMPs) are produced predominantly by the *Drosophila* fat body via the *Toll* and *Imd* pathways (De Gregorio et al., 2002). As described previously in Chapter 4, a loss of *fs(1)h* in the fat body increased the expression levels of AMPs (Figure 4-4 and 4-5). We wanted to further assay the effect of *foxo* heterozygosity on AMP expression in flies with *fs(1)h* knockdown in the fat body. In line with our previous finding of *foxo* heterozygosity rescuing the uninfected survival, we found that AMP expression was nearly completely normalised in uninfected *fs(1)h* knockdown flies also lacking one copy of *foxo* (Figure 5-3A-G). Loss of one copy of *foxo* in the control flies had little effect on AMP expression. We were also able to observe the same reduction in AMP expression when dissected fat body samples were obtained (Figure 5-4A-G). We went on to analyse AMP expression in infected flies that had the loss of *fs(1)h* in the fat body along with the reduction in *foxo.* AMP expression was still significantly elevated in these flies following bacterial infection with *F. novicida* (Figure 5-5A-G). The elevations in AMP expression levels were also shown following *L. monocytogenes* infection in a number of cases (Figure 5-5A, B, E and F). Reducing the level of *foxo* could not rescue the overproduction of the AMPs analysed after bacterial infection when flies are lacking *fs(1)h* in the fat body. These data indicate that *fs(1)h* may be affecting baseline and infection-induced AMP expression via distinct mechanisms.

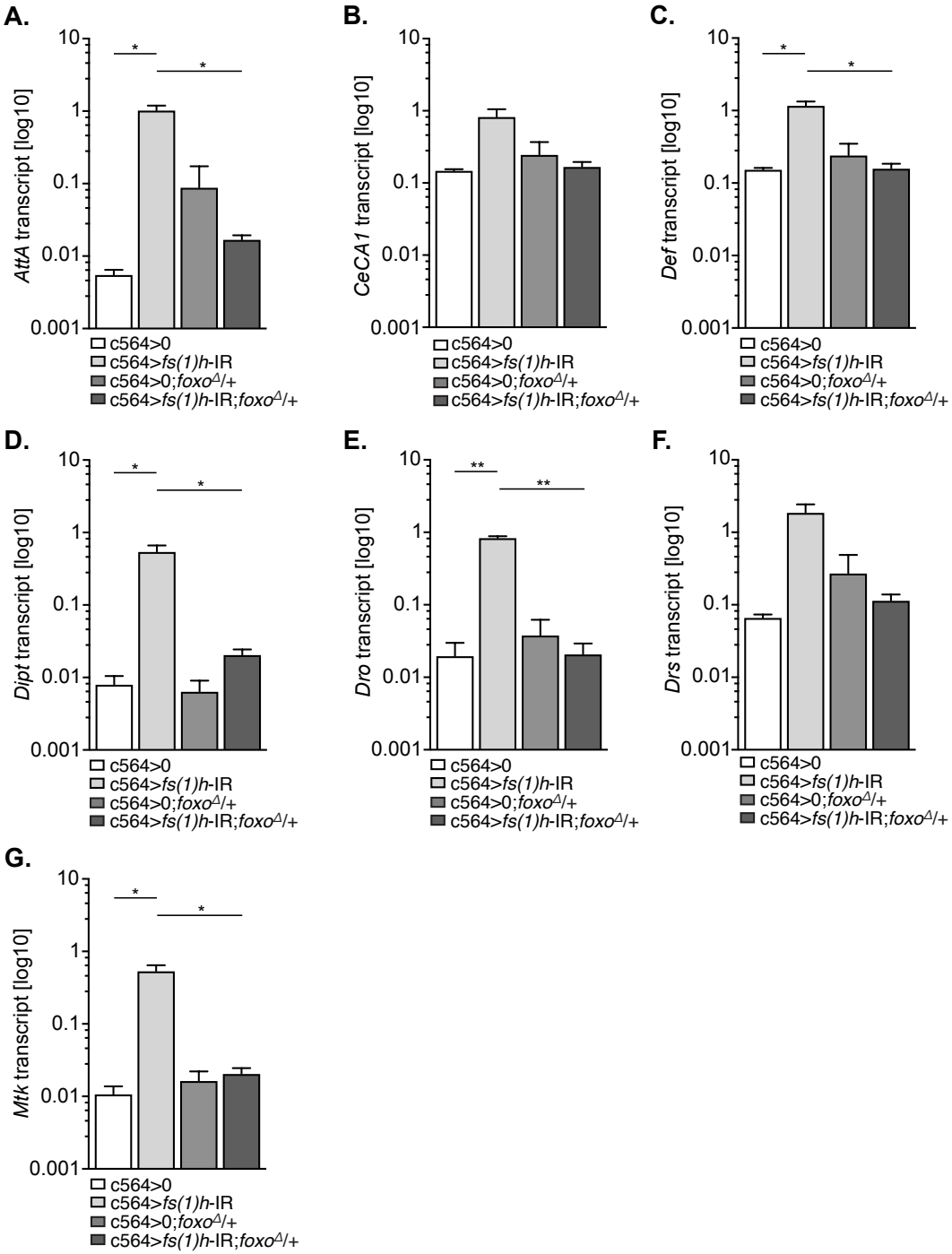


Figure 5-3: AMP levels of whole fly samples were rescued when one copy of *foxo* was removed from *fs(1)h* knockdown flies

RT-qPCR analysis of 5-7 day old whole fly samples following knockdown of *fs(1)h* in the fat body (*c564>fs(1)h*-IR, light grey bars), control samples (*c564>*0, white bars), *fs(1)h* fat body knockdowns heterozygous for *foxo* (*c564>fs(1)h*-IR;*foxo*$^{\Delta}$/+, dark grey bars), and control samples heterozygous for *foxo* (*c564>*0;*foxo*$^{\Delta}$/+, grey bars). All RT-qPCR data was normalised to the housekeeping gene, α*-tubulin.* Data shown on log10 scale. Normalised transcript levels of **A.** *Attacin,* **B.** *Cecropin A1,* **C.** *Defensin,* **D.** *Diptericin,* **E.** *Drosocin,* **F.** *Drosomycin* and **G.** *Metchnikowin.* Unpaired t tests, *p<0.05, **p<0.01, no stars indicate lack of statistical significance. Data represented as mean of n=8 biological repeats per genotype (3 flies per sample), error bars as SEM.

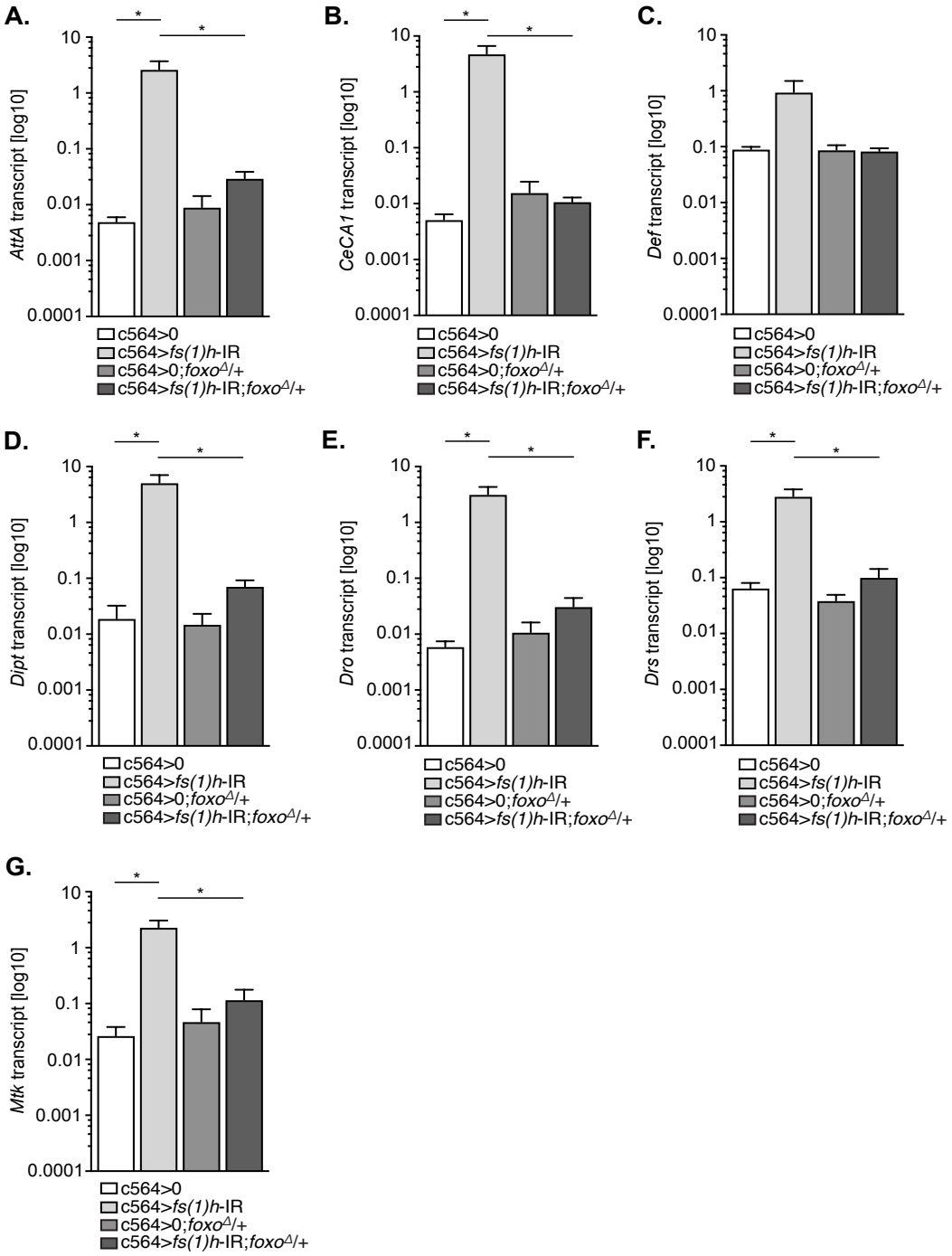


Figure 5-4: AMP transcript levels of dissected fat body samples were rescued when one copy of *foxo* was removed from *fs(1)h* knockdown flies

RT-qPCR analysis of 5-7 day old dissected fat body samples following knockdown of *fs(1)h* in the fat body (*c564>fs(1)h*-IR, light grey bars), control samples (*c564*>0, white bars), *fs(1)h* fat body knockdowns heterozygous for *foxo* (*c564>fs(1)h*-IR;*foxo*$^{\Delta}$/+, dark grey bars), and control samples heterozygous for *foxo* (*c564*>0;*foxo*$^{\Delta}$/+, grey bars). All RT-qPCR data was normalised to the housekeeping gene, α-*tubulin.* Data shown on a log10 scale. Normalised transcript levels of **A.** *Attacin,* **B.** *Cecropin A1,* **C.** *Defensin,* **D.** *Diptericin,* **E.** *Drosocin,* **F.** *Drosomycin* and **G.** *Metchnikowin.* Unpaired t tests, *p<0.05, **p<0.01, no stars indicate lack of statistical significance. Data represented as mean of n=8 biological repeats per genotype (3 flies per sample), error bars as SEM.

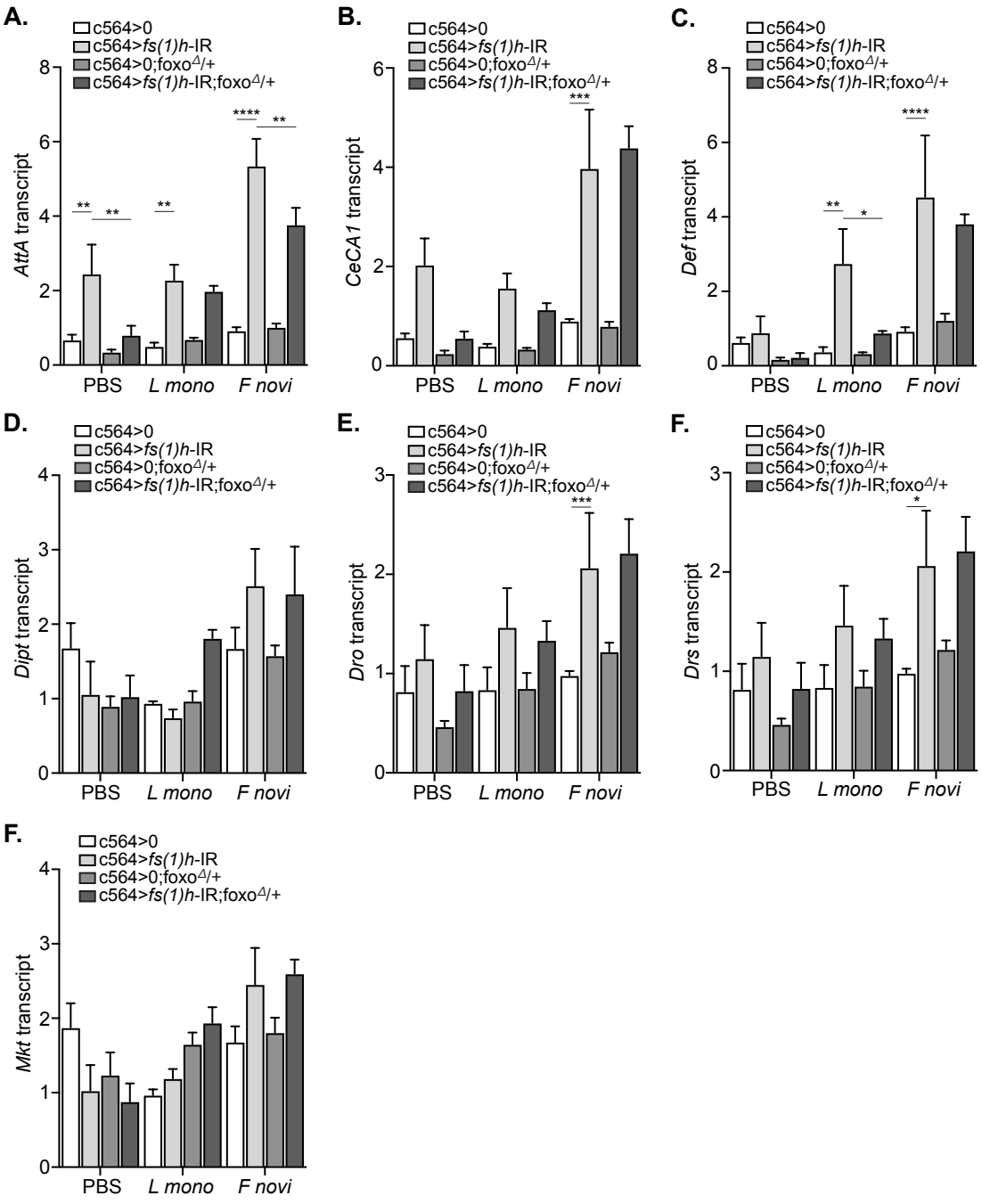


Figure 5-5: Infected fly AMP expression was not rescued by foxo heterozygosity
RT-qPCR analysis of 5-7 day old whole fly samples following knockdown of *fs(1)h* in the fat body (*c564>fs(1)h*-IR, light grey bars), control samples (*c564*>0, white bars), *fs(1)h* fat body knockdowns heterozygous for *foxo* (*c564>fs(1)h*-IR;*foxo*$^{\Delta}$/+, dark grey bars), and control samples heterozygous for *foxo* (*c564*>0;*foxo*$^{\Delta}$/+, grey bars). Injected with PBS, as a wounding control, *Listeria monocytogenes* or *Francisella novicida.* All RT-qPCR data was normalised to the housekeeping gene, α-*tubulin*. Normalised transcript levels of **A.** *Attacin,* **B.** *Cecropin A1,* **C.** *Defensin,* **D.** *Diptericin,* **E.** *Drosocin,* **F.** *Drosomycin* and **G.** *Metchnikowin.* 2-way ANOVA, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, no stars indicate lack of statistical significance. Data represented as mean of n=5 biological repeats per genotype and injection group (3 flies per sample), error bars as SEM.

5.4.1. *foxo* heterozygosity normalised total Relish protein levels

As shown in Chapter 4, Relish, the NF-κB family member responsible for *Imd*-dependent antimicrobial peptide expression was increased following *fs(1)h* knockdown in the fat body. Here, we wanted to investigate whether a reduction in *foxo* levels in *fs(1)h* fat body knockdown flies had an effect on the abundance of the Relish protein in the flies following bacterial infection, but also when unchallenged. We found the protein levels of Relish were normalised back to the control level by removing a single copy of *foxo* in *fs(1)h* knockdown flies in uninfected conditions (Figure 5-6A). Reducing the level of *foxo* alone did not affect the abundance of total Relish protein, nor did it alter the activation of Relish, shown by the unchanged levels of C-terminal fragment of Relish (Figure 5-6A). The normalisation of Relish protein level fitted with the normalisation of uninfected AMP expression in the flies with a reduction in *foxo*.

Flies with the loss of *fs(1)h* in the fat body and one copy of *foxo* removed had normalised levels of total Relish protein following injection with PBS, as a wounding control, or following infection with *F. novicida* (Figure 5-6B). Furthermore, there was no change in Relish activation level, indicated by the amount of the C-terminal fragment, following the removal of *fs(1)h* in the fat body and one copy of *foxo* (Figure 5-6B). After PBS injection or infection with *F. novicida,* we detected an increase in the level of the C-terminal fragment when *fs(1)h* was knocked down in the fat body compared to controls. These findings fit with the reduced survival and the increased AMP levels following bacterial infections. *foxo* heterozygosity was able to completely abolish the overexpression of Relish post-infection, but was unable to rescue the survival or AMP expression levels.

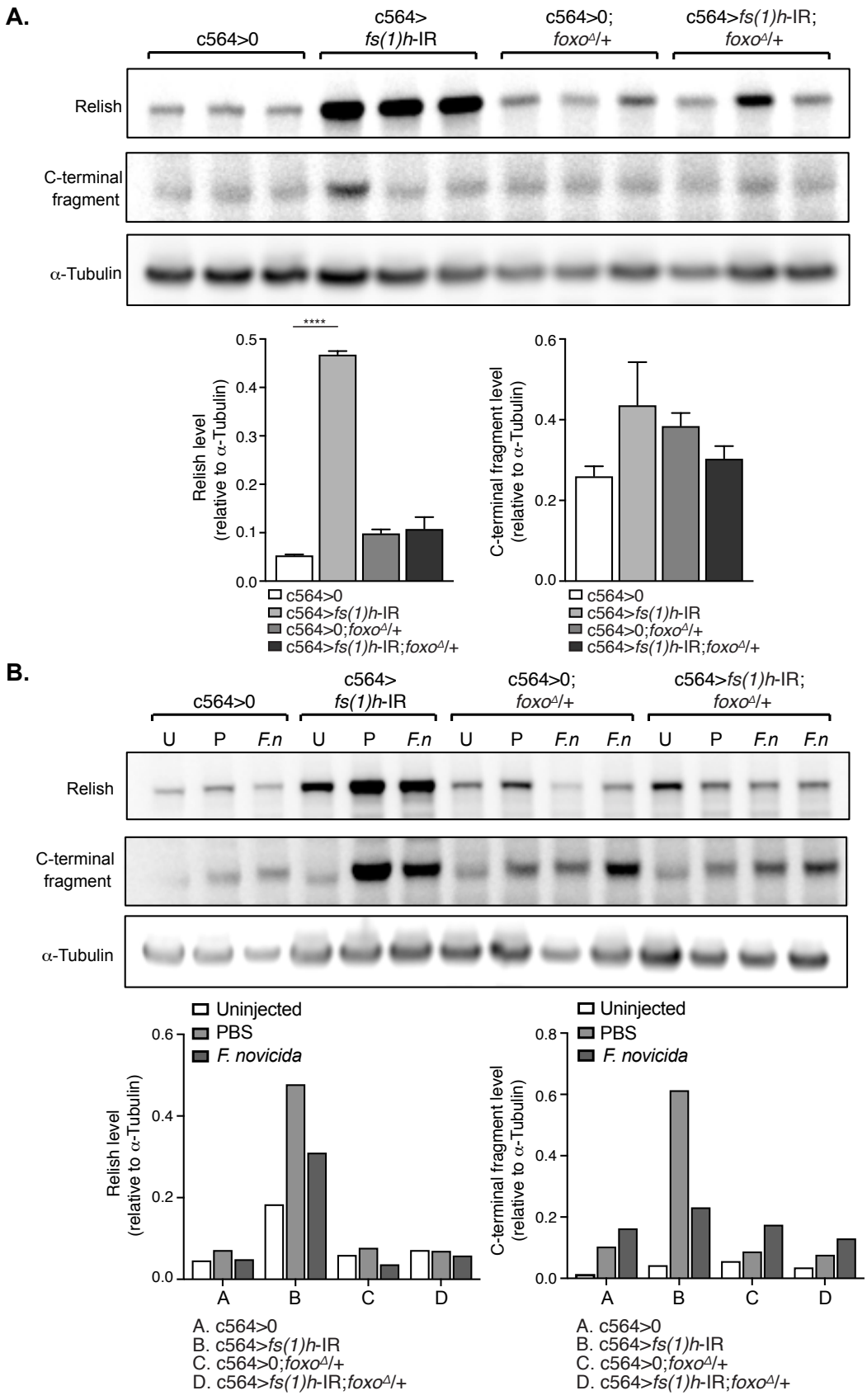


Figure 5-6: Protein levels of Relish were rescued following the removal of one copy of *foxo* in *fs(1)h* knockdown flies

Western blot analysis of 5-7 day old fly samples following knockdown of *fs(1)h* in the fat body (*c564>fs(1)h*-IR), *fs(1)h* fat body knockdowns heterozygous for *foxo* (*c564>fs(1)h*-IR;*foxo*$^{\Delta}$/+),

and control samples (*c564*>0 and *c564*>0;*foxo*$^{\Delta}$/+). **A.** Relish protein level following *fs(1)h* knockdown in the fat body (light grey bars), *fs(1)h* fat body knockdowns heterozygous for *foxo* (dark grey bars), control samples (white bars), and control samples heterozygous for *foxo* (grey bars). Values represented as intensity relative to *α-tubulin*. Data shown as mean + SEM, ****p<0.0001, no stars indicate lack of statistical significance. **B.** Relish protein level following bacterial infection of *fs(1)h* knockdown in the fat body (B), *fs(1)h* fat body knockdowns heterozygous for *foxo* (D), control samples (A), and control samples heterozygous for *foxo* (C). Uninjected samples (white bars), injected with PBS (light grey bars) or injected with *Francisella novicida* (dark grey bars). U; uninjected, P; PBS injected, F.n; F. novicida infected. Values represented as intensity relative to *α-tubulin*, data shown as mean.

5.5. The *fs(1)h* starvation phenotype was rescued by reducing *foxo* levels

The transcription factor *foxo* plays important roles in insulin signalling particularly when energy levels are low (Kramer et al., 2003). Therefore, we wanted to investigate if a reduction in *foxo* levels could also rescue the metabolic phenotypes we found following *fs(1)h* knockdown in the fat body. The reduction in survival that was observed when flies were lacking *fs(1)h* in the fat body were put onto starvation food was rescued back to the control genotype level by the removal of one copy of *foxo* (Figure 5-7). This finding may indicate that *foxo* was playing an important role in the metabolic phenotypes we observe in flies with a deficiency of *fs(1)h* in the fat body.

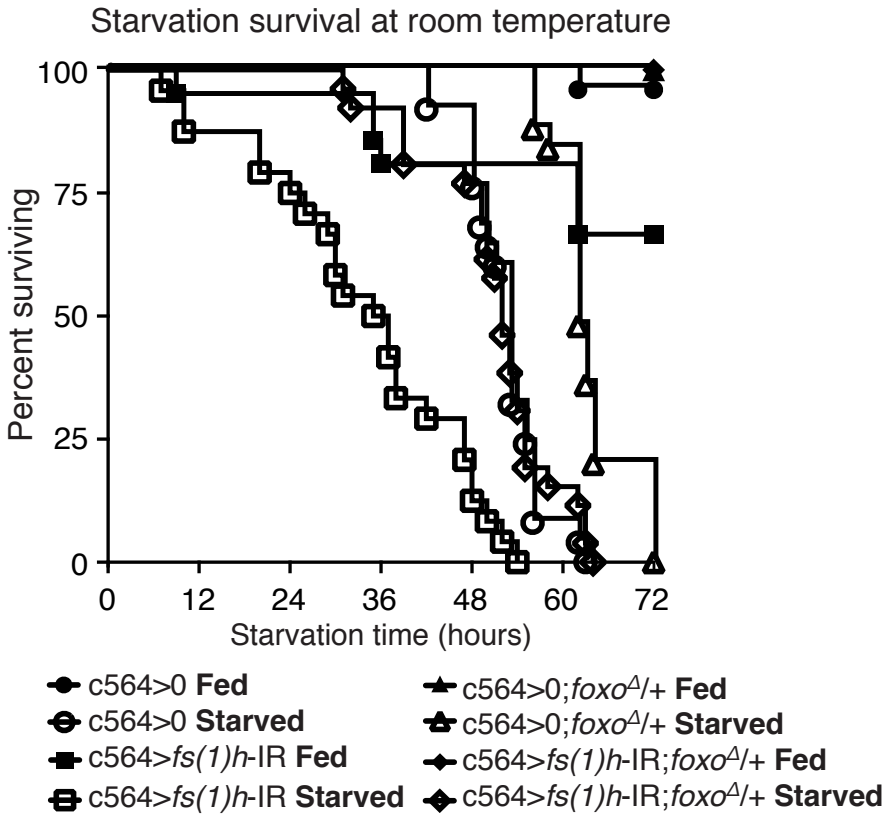


Figure 5-7: Removing one copy of *foxo* in *fs(1)h* knockdown flies rescued the starvation phenotype

Starvation survival of 5-7 day old flies with *fs(1)h* knocked down in the fat body (*c564>fs(1)h*-IR, squares), *fs(1)h* fat body knockdowns heterozygous for *foxo* (*c564>fs(1)h*-IR;*foxo*$^{\Delta}$*/+,* diamonds), and control samples (*c564*>0, circles and *c564*>0;*foxo*$^{\Delta}$/+, triangles). Fed flies (closed shapes) and starved flies (open shapes) were counted every 2 hours during the day for deaths and percent survival was calculated, n=20 per group.

5.5.1. Flies heterozygous for *foxo* were able to utilise triglyceride stores

In both mammals and *Drosophila,* foxo regulates the transcription of lipases and other enzymes and transcription factors in adipose tissue that are required for lipid droplet lipolysis (Karpac et al., 2013). Fluctuations in the insulin-signalling pathway are also associated with abnormal lipid metabolism (Saltiel and Kahn, 2001). To identify if the reduction of *foxo* can also rescue the defect in lipid storage mobilisation, we assayed the quantity of triglycerides in *fs(1)h* knockdown flies when the level of *foxo* was also reduced. Due to the functional roles of foxo in regulating the expression of lipid metabolism genes (Gross et al., 2008), *foxo* heterozygosity caused an increase in total triglyceride level whether *fs(1)h* was knocked down in the fat body or in combination with the control genotype. However, flies lacking f*s(1)h* in the fat body and lacking one copy of *foxo* regained their ability to utilise their triglyceride stores following 24-hours starvation (Figure 5-8). Therefore, we conclude that elevated *foxo* activity was responsible for the defect in energy mobilisation we identified following the loss of *fs(1)h* in the fat body.

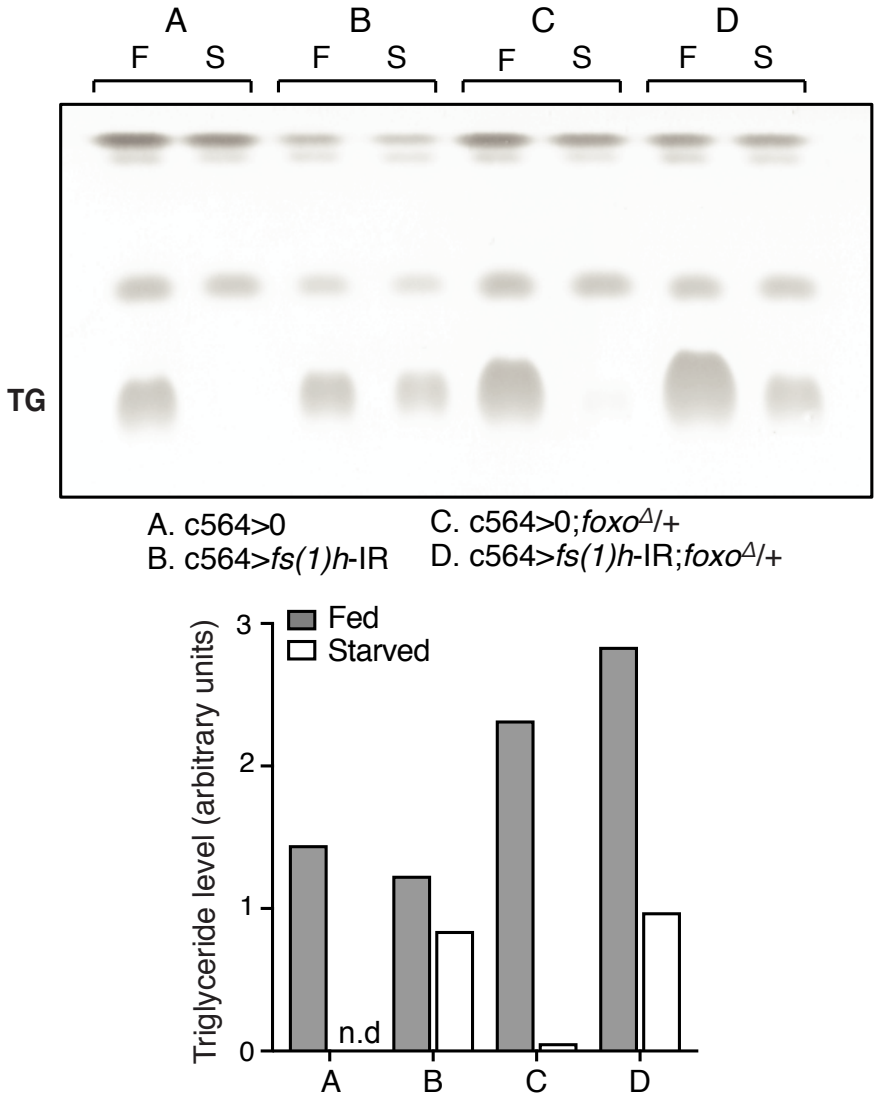


Figure 5-8: Triglycerides could be utilised by removing one copy of *foxo* in *fs(1)h* knockdown flies

Thin layer chromatography (TLC) analysis of 5-7 day old total fly triglyceride levels following *fs(1)h* knockdown in the fat body (*c564>fs(1)h*-IR, B), *fs(1)h* fat body knockdowns heterozygous for *foxo* (*c564>fs(1)h*-IR;*foxo*$^{\Delta}$/+, D), and control samples (*c564*>0, A and *c564*>0;*foxo*$^{\Delta}$/+, C). Fed conditions (grey bars) and following 24-hour starvation (white bars). TLC plate shows one fed and one starved group for each genotype. F; fed, S; starved and TG; triglyceride, n.d; non-detected.

5.5.2. Transcript levels of lipid metabolism genes were restored to control levels when one copy of *foxo* was removed

FOXO plays important roles in regulating the transcription of a number of lipid metabolism genes (Gross et al., 2008). Following fat body knockdown of *fs(1)h,* there were reductions in the transcript levels of *bmm, plin1* and *plin2*, three genes that are known to be important in the mobilisation of triglycerides. Levels of *Hsl* showed no change when *fs(1)h* was knocked down in the fat body, but was slightly upregulated when *foxo* levels were reduced in flies with *fs(1)h* knockdown in both whole fly and dissected fat body samples (Figure 5-9A and A'). Transcript levels of *bmm* in whole fly samples showed no changes across any of the genotypes measured, whereas the reduction of *bmm* seen in the dissected fat body samples was rescued when one copy of *foxo* was removed (Figure 5-9B and B'). *plin1* was significantly upregulated when *foxo* levels were reduced in the *fs(1)h* knockdown flies in both the whole fly and dissected fat body samples (Figure 5-9C and C'), and *plin2*, similarly to *plin1,* was also upregulated when flies were heterozygous for *foxo* (Figure 5-9D and D'). Here, we show that in flies deficient for *fs(1)h* in the fat body, removing one copy of *foxo* rescued the reduced levels of genes involved in lipid metabolism back to the same level as the control group. This rescue in gene expression may fit with their ability to withstand 24-hour starvation and the ability to utilise stored triglycerides upon starvation.

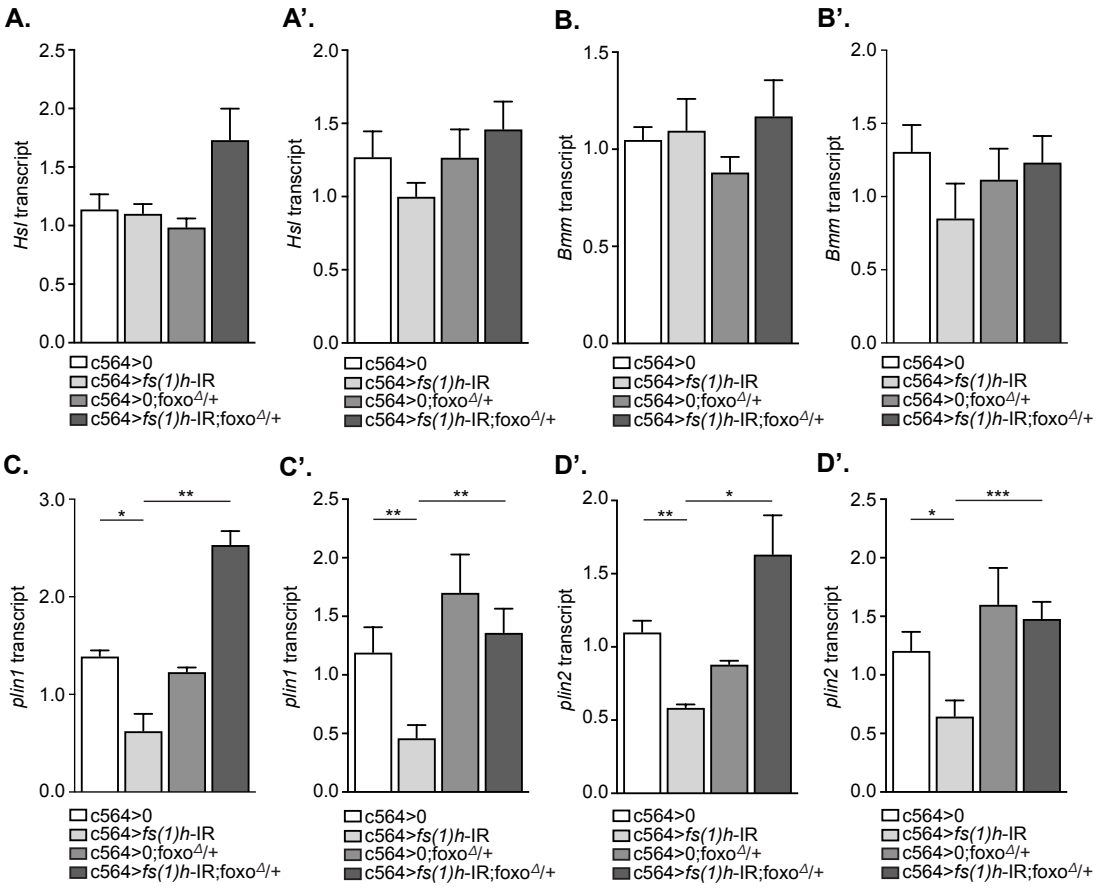


Figure 5-9: Lipid metabolism genes showed variation when one copy of *foxo* was removed

RT-qPCR analysis of 5-7 day old whole flies and dissected fat body samples following *fs(1)h* knockdown in the fat body (*c564>fs(1)h*-IR, light grey bars), *fs(1)h* fat body knockdowns heterozygous for *foxo* (*c564>fs(1)h*-IR;*foxo*$^{\Delta}$*/+,* dark grey bars), and control samples (*c564*>0, while bars and *c564*>0;*foxo*$^{\Delta}$*/+*, grey bars). All RT-qPCR data was normalised to the housekeeping gene, α*-tubulin*. Normalised transcript levels of **A.** *Hormone sensitive lipase* (*Hsl*) in whole fly samples, **A.'** *Hsl* in dissected fat body samples, **B.** *brummer* (*bmm*) in whole fly samples, **B.'** *bmm* in dissected fat body samples, **C.** *perilipin 1* (*plin1*) in whole fly samples, **C.'** *plin1* in dissected fat body samples, **D.** *perilipin 2* (*plin2*) in whole fly samples and **D.'** *plin2* in dissected fat body samples. Unpaired t tests, *p<0.05, **p<0.01, ***p<0.001, no stars indicate lack of statistical significance. Data represented as mean of n=8/10 biological repeats per genotype (3 flies per sample), error bars as SEM.

5.6. Circulating sugar levels were restored when one copy of *foxo* was removed in flies lacking *fs(1)h* in the fat body

In mammals, *foxo* is thought to promote hepatic glucose production by a variety of mechanism and in turn contribute to glucose homeostasis (Nakae et al., 2008). Flies lacking *fs(1)h* in the fat body showed low levels of the circulating sugars, trehalose and glucose and low levels of the stored carbohydrate, glycogen. Levels of trehalose and glucose were normalised back to the control level in *fs(1)h* knockdown flies also lacking one copy of *foxo* (Figure 5-10A, B), while glycogen levels were still reduced in these flies (Figure 5-10C). These data imply that a reduction in *foxo* restores circulating sugar levels when *fs(1)h* was lost in the fat body, but was unable to rescue the glycogen level suggesting the glycogen homeostasis may be independent of *foxo* level.

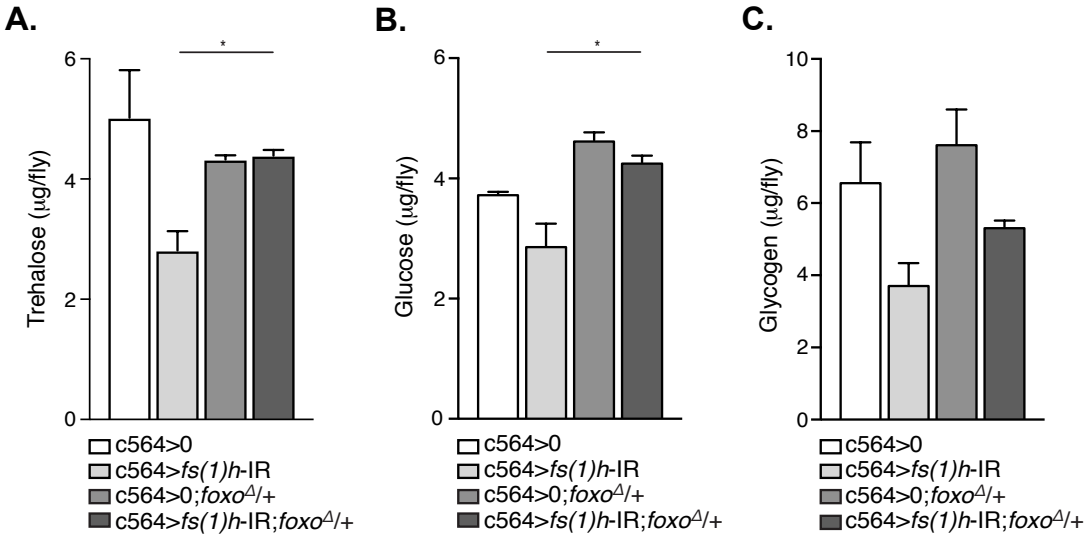


Figure 5-10: Circulating sugar levels were rescued in flies heterozygous for *foxo*
Trehalose, glucose and glycogen content in 5-7 day old male flies following *fs(1)h* knockdown in the fat body (*c564>fs(1)h*-IR, light grey bars), *fs(1)h* fat body knockdowns heterozygous for *foxo* (*c564>fs(1)h*-IR;*foxo*$^{\Delta}$*/+,* dark grey bars), and control samples (*c564*>0, while bars and *c564*>0;*foxo*$^{\Delta}$*/+*, grey bars). Circulating sugars of **A.** Trehalose*,* **B.** Glucose*,* and stored carbohydrate levels of **C.** Glycogen. Unpaired t tests, *p<0.05, no stars indicate lack of statistical significance. Data represented as mean of n=6 biological repeats per genotype (3 flies per sample), error bars as SEM.

5.7. Activated AKT levels were rescued in flies lacking *fs(1)h* in the fat body and heterozygous for *foxo*

The AKT kinase of the insulin pathway has two major known metabolic target molecules, FOXO and TOR. AKT phosphorylates FOXO leading to its inactivation, while it activates the TOR complex and its downstream targets including S6 kinase (S6K), which when phosphorylated activates protein synthesis (Magnuson et al., 2012). We found decreased phosphorylated AKT and S6 kinase levels following the loss of *fs(1)h* in the fat body. As described previously, we assumed the decrease in phosphorylated AKT following *fs(1)h* knockdown in the fat body resulted in elevated *foxo* levels as indicated by the upregulation of *foxo* target genes (Figure 4-17). We wanted to further investigate whether removing one copy of *foxo* would also rescue the activated AKT and S6K levels. Interestingly, we found that activated AKT (Figure 5-11A) and S6 kinase (Figure 5-11B) levels were both rescued in *fs(1)h* knockdown flies when they were also heterozygous for *foxo*. Furthermore, we wanted to ensure that the changes in the activity of the insulin-signalling pathway were not causing the development of insulin resistance in these flies. Heterozygous *foxo* mutants with a loss of *fs(1)h* in the fat body did not show any sign of insulin resistance (Figure 5-11C). These data suggest that a reduction of *foxo* can rescue the reduced levels of activated AKT and S6K in flies deficient for *fs(1)h* in the fat body.

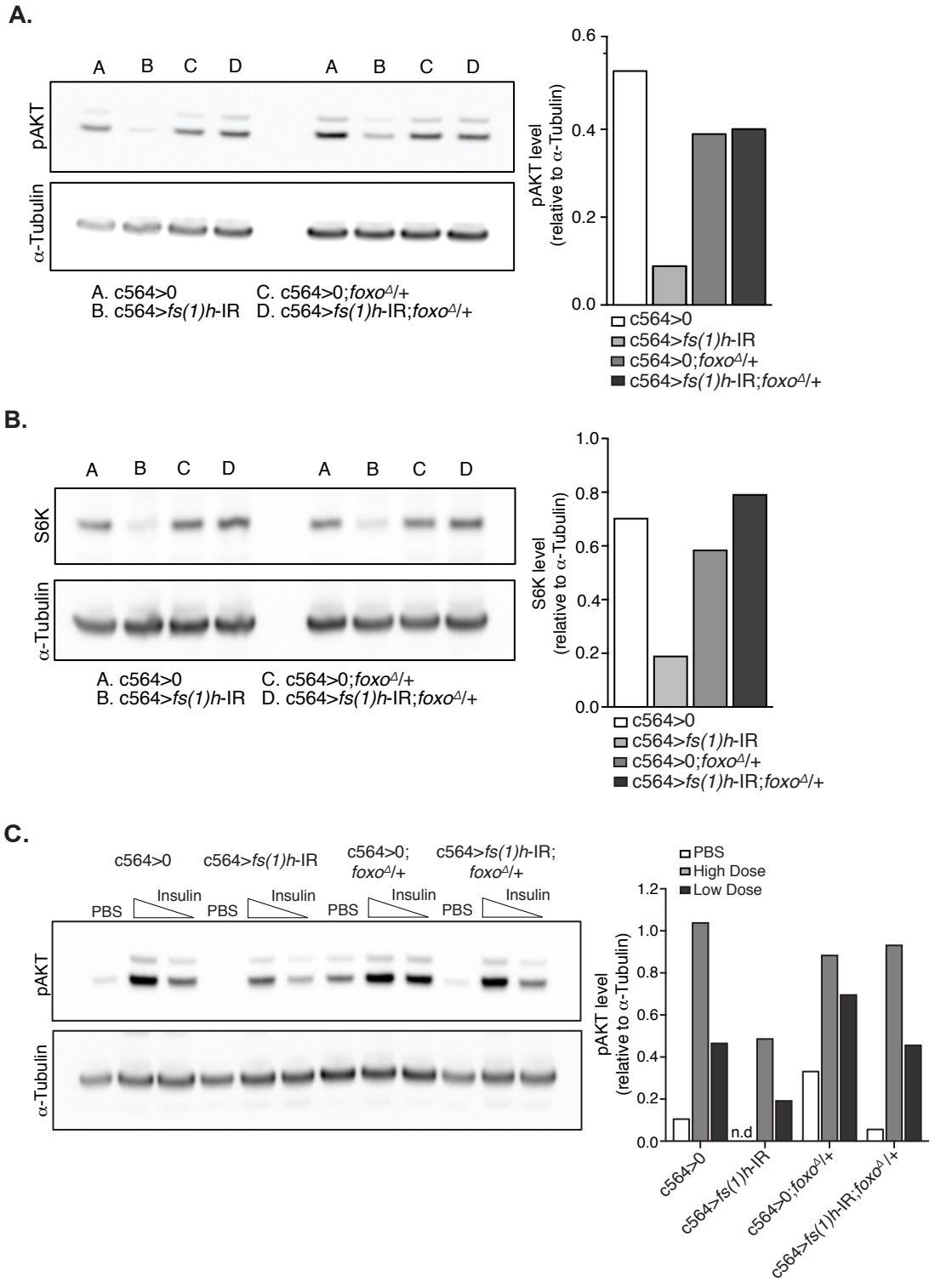


Figure 5-11: Activated AKT and S6 Kinase levels were rescued in *foxo* heterozygous flies with *fs(1)h* knocked down the fat body

Western blot analysis of 5-7 day old flies following knockdown of *fs(1)h* in the fat body (*c564>fs(1)h*-IR), *fs(1)h* fat body knockdowns heterozygous for *foxo* (*c564>fs(1)h*-IR;*foxo*$^{\Delta}$/+), and control samples (*c564*>0 and *c564*>0;*foxo*$^{\Delta}$/+). **A.** AKT Ser505 phosphorylation in control (white bars, grey bars), *fs(1)h* fat body knockdown (light grey bars) and *fs(1)h* fat body knockdowns heterozygous for *foxo* (dark grey bars) flies. Values represented as intensity relative to *α-tubulin*, data shown as mean. **B.** p70 S6 Kinase (S6K) in control groups (white bars, grey bars), *fs(1)h* fat body knockdown flies (light grey bars) and flies with *fs(1)h* fat body knockdowns heterozygous for *foxo* (dark grey bars). Values represented as intensity relative to

α-tubulin, data shown as mean. **C.** To assay insulin sensitivity, AKT phosphorylation levels were measured following injection with PBS (white bars) or a high (320pg/fly; light grey bars) or low (64 pg/fly; dark grey bars) dose of human insulin. Values represented as intensity relative to *α-tubulin*, data shown as mean.

5.8. Expression of *upd2* was restored following a reduction in *foxo* levels in *fs(1)h* fat body knockdown flies

Expression of secreted insulin-regulatory factors were assayed in flies with *fs(1)h* knocked down in the fat body along with *foxo* heterozygosity. *upd1 and upd2,* two ligands of the Jak/STAT pathway, are both thought to be analogous to mammalian leptin (Beshel et al., 2017; Rajan and Perrimon, 2012). Interestingly, we found the increase of *upd1* in the *fs(1)h* knockdown flies was further increased when one copy of *foxo* was removed in whole fly samples (Figure 5-12A). However, in the dissected fat body samples there was little change in the transcript level of *upd1* (Figure 5-12A'). The upregulation of *upd2* transcript level that was observed following *fs(1)h* knockdown in the fat body was rescued by the *foxo* heterozygosity in whole fly (Figure 5-12B) and dissected fat body (Figure 5-12B') samples. Here, we show that cytokine signals with insulin-regulatory functions in the brain could be rescued back to control levels in dissected fat body samples, however *upd1* was not rescued in whole fly samples. This may indicate that *upd2,* as a fat body-derived signal, was affected by the fs(1)h/FOXO axis. This observation fits with reports showing *upd2* is a cytokine signal released from and regulated by energy storage in the fat body (Rajan and Perrimon, 2012).

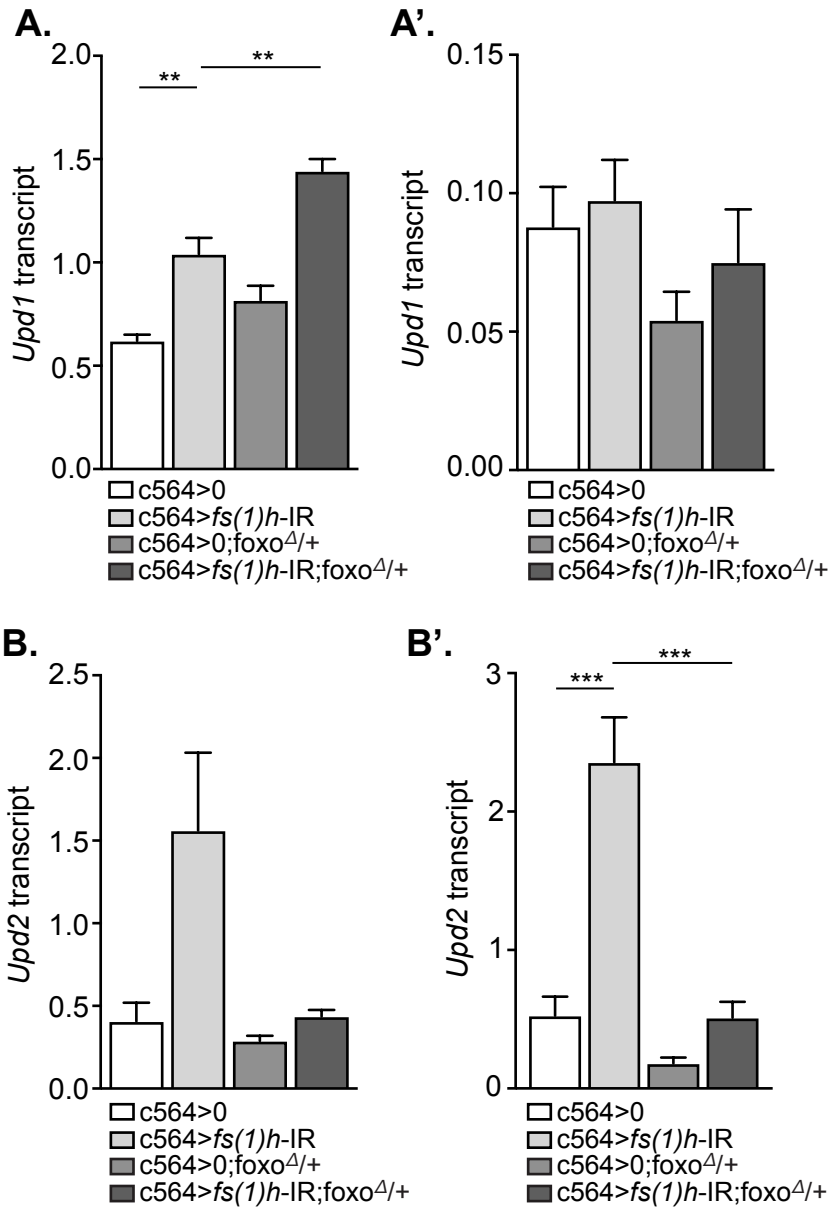


Figure 5-12: *upd1* and *upd2* transcript levels were altered following the reduction of *foxo* in *fs(1)h* knockdown flies

RT-qPCR analysis of 5-7 day old whole flies and dissected fat body samples following knockdown of *fs(1)h* in the fat body (*c564>fs(1)h*-IR, light grey bars), *fs(1)h* fat body knockdowns heterozygous for *foxo* (*c564>fs(1)h*-IR;*foxo*$^{\Delta}$*/+,* dark grey bars), and control samples (*c564*>0, white bars and *c564*>0;*foxo*$^{\Delta}$/+, grey bars). All RT-qPCR data was normalised to the housekeeping gene, α-*tubulin*. Normalised transcript levels of **A.** *unpaired 1* (*upd1*) in whole fly samples, **A.'** *upd1* in dissected fat body samples, **B.** *unpaired 2* (*upd2*) in whole fly samples and **B.'** *upd2* in dissected fat body samples. Unpaired t tests, **p<0.01, ***p<0.001, no stars indicate lack of statistical significance. Data represented as mean of n=8/10 biological repeats per genotype (3 flies per sample), error bars as SEM.

5.9. Nutrient-responsive signals showed variation in *foxo* heterozygous flies with *fs(1)h* knocked down in the fat body.

The *Drosophila* fat body plays a central role in nutrient sensing mechanisms, in response to intracellular amino acids, lipids or sugars, the fat body can regulate systemic growth in response to nutritional changes (Colombani et al., 2003). *Gbp1 and Gbp2* are both regulated by amino acid levels, whereas *CCHa2* is regulated by sugar and lipid levels (Koyama and Mirth, 2016). *ImpL2,* is a secreted insulin antagonist that inhibits DILP activity, is expressed in the ring gland of the brain and can sense changes nutritional state (Sarraf-Zadeh et al., 2013). *Gbp1*, showed no change in transcript level in any of the genotypes measured in whole fly samples (Figure 5-13A). However, in dissected fat body samples, there was a significant reduction in *Gbp1* levels when *fs(1)h* was knocked down in the fat body. Surprisingly, *Gbp1* was still reduced and not rescued by *foxo* heterozygosity (Figure 5-13A'). *Gbp2* was significantly reduced following *fs(1)h* knockdown in the fat body, however, this phenotype was rescued by removing one copy of *foxo* in whole fly samples (Figure 5-13B) and dissected fat body samples (Figure 5-13B'). *CCHa2* levels were significantly reduced following *fs(1)h* knockdown in the fat body, however *w*hole fly sample *CCHa2* transcript levels remained low in *foxo* heterozygous flies (Figure 5-13C). Interestingly, this result was partially rescued in dissected fat body samples (Figure 5-13C'). The transcript level of *ImpL2* showed no differences in whole fly samples of any genotype measured (Figure 5-13D). In dissected fat body samples, *fs(1)h* knockdown in the fat body reduced *ImpL2* levels, which was partly rescued by removing one copy of *foxo* (Figure 5-13D'). We found that nutrient sensing gene levels could be partly rescued by the reduction in *foxo* level in the *fs(1)h* knockdown flies. However, we assume *Gbp1* may be regulated by another mechanism and the change observed following *fs(1)h* knockdown in the fat body may be *foxo*-independent.

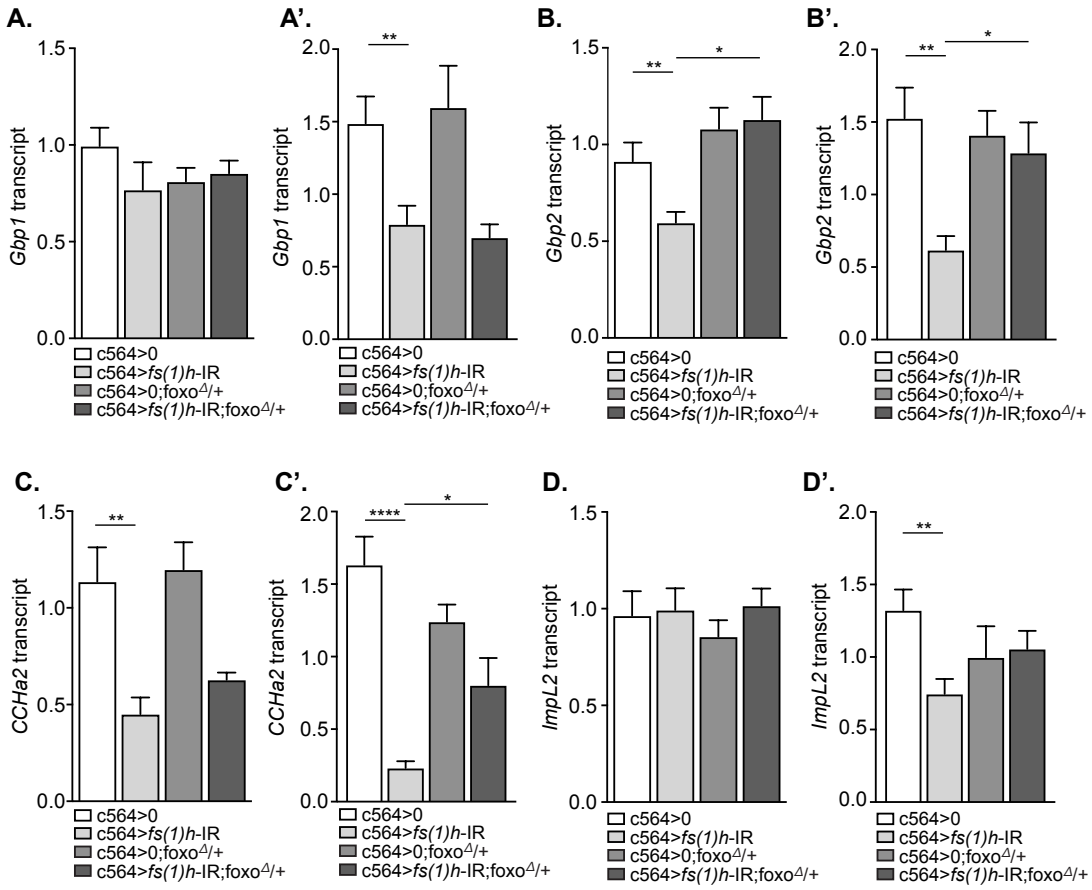


Figure 5-13: Removing a copy of *foxo* in the *fs(1)h* knockdown flies regulated insulin-derived signals

RT-qPCR analysis of 5-7 day old whole flies and dissected fat body following knockdown of *fs(1)h* in the fat body (*c564>fs(1)h*-IR, light grey bars), *fs(1)h* fat body knockdowns heterozygous for *foxo* (*c564>fs(1)h*-IR;*foxo$^{\Delta}$/+,* dark grey bars), and control samples (*c564*>0, white bars and *c564*>0;*foxo$^{\Delta}$/+*, grey bars). All RT-qPCR data was normalised to the housekeeping gene, α*-tubulin*. Normalised transcript levels of **A.** *Growth-blocking peptide 1* (*Gbp1*) in whole fly samples, **A'.** *Gbp1* in dissected fat body samples, **B.** *Growth-blocking peptide 2* (*Gbp2*) in whole fly samples, **B'.** *Gbp2* in dissected fat body samples, **C.** *CCHamide-2* (CCHa2) in whole fly samples, **C'.** *CCHa2* in dissected fat body samples, **D.** *Ecdysone-inducible gene L2* (*ImpL2*) in whole fly samples and **D'.** *ImpL2* in dissected fat body samples**.** Unpaired t tests, *p<0.05, **p<0.01, ****p<0.0001, no stars indicate lack of statistical significance. Data represented as mean of n=8/10 biological repeats per genotype (3 flies per sample), error bars as SEM.

5.10. Knockdown of *fs(1)h* in the fat body increased FOXO^{GFP} levels and caused potential ubiquitin-proteasome dysfunction

Due to the phenotypes observed, we wanted to investigate whether fs(1)h was regulating FOXO protein levels. Therefore, we used a transgenic fly line carrying a FOXO^{GFP} fusion protein regulated by the endogenous foxo locus. We combined this fly line with *fs(1)h* knockdown in the fat body to measure the amount of FOXO in flies with *fs(1)h* knockdown in the fat body. We wanted to assay the amount of FOXO^{GFP} in flies with *fs(1)h* knocked down in the fat body, here we could show there was an increased amount of FOXO^{GFP} (Figure 5-14A). However, in these samples there was also a genotype specific dysregulation of *α-tubulin*, which was used as the loading control. A second loading control, GAPDH, showed a similar dysregulated pattern (data not shown). Therefore, we assumed that *fs(1)h* knockdown may be affecting the abundance of proteins in the flies and possibly regulating the degradation process of proteins within the cells.

One mechanism that regulates protein degradation is ubiquitination of proteins that are targeted to the proteasome and degraded (Lecker et al., 2006). Ubiquitin is a highly conserved protein present in all eukaryotes as a free polypeptide or joined to a range of cytoplasmic, nuclear and cell surface proteins (Lee et al., 1988). Ubiquitination is a post-translational modification involved in regulating functions such as protein degradation and signal transduction (Taylor and Jobin, 2005; Wilkinson, 1987) by ubiquitin attachment to other cellular proteins, thus changing the stability, localisation or activity of a target protein (Pickart and Eddins, 2004). The ubiquitin proteasome pathway is required to degrade most short-lived proteins, for example proteins unable to fold within the endoplasmic reticulum (Araki and Nagata, 2011) or cell cycle proteins, whose destruction at the correct time is vital during cell division (Reed, 2006). A specific ubiquitin complex has been shown to regulate the *NF-κB* pathway, *NF-κB* is vital for inflammatory and immune responses and abnormal *NF-κB* signalling is implemented in many diseases (Tokunaga, 2013). Here, we could show that flies with *fs(1)h* knockdown in the fat body also displayed an increase in protein ubiquitination compared to the control groups (Figure 5-14B). In line with this, Coomassie staining showed there were variations in the proteins detected and the quantity of these proteins in samples containing the *fs(1)h* knockdown (Figure 5-14C). We could conclude that the loss of *fs(1)h* in the fat body led to elevated levels of the FOXO protein. We also found a potential increase in ubiquitination and protein level when *fs(1)h* was removed from the *Drosophila* fat body.

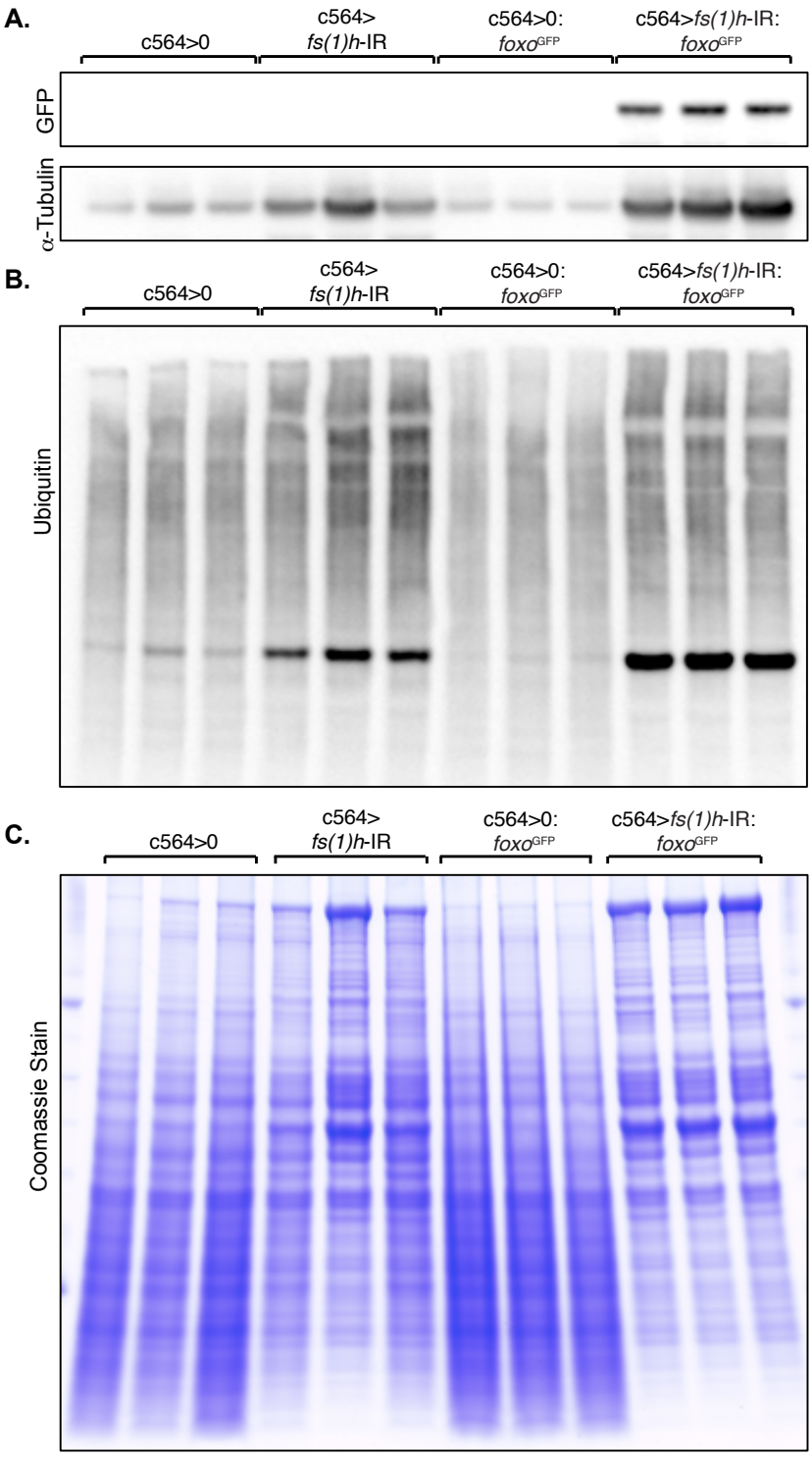


Figure 5-14: Using FOXO^{GFP} to investigate an interaction between FOXO and fs(1)h
Western blot analysis of 5-7 day old flies following knockdown of *fs(1)h* in the fat body (*c564>fs(1)h*-IR), *fs(1)h* fat body knockdowns heterozygous for *foxo* (*c564>fs(1)h*-IR;*foxo*$^{\Delta}$/+), and control samples (*c564*>0 and *c564*>0;*foxo*$^{\Delta}$/+). **A.** GFP protein level and *α-tubulin* in controls, *fs(1)h* fat body knockdown and *fs(1)h* fat body knockdowns heterozygous for *foxo* flies. **B.** Ubiquitin protein level in controls, *fs(1)h* fat body knockdown and *fs(1)h* fat body knockdowns heterozygous for *foxo* flies. **C.** Coomassie stain for controls, *fs(1)h* fat body knockdown and *fs(1)h* fat body knockdowns heterozygous for *foxo* flies.

5.11. *fs(1)h* transcript levels were elevated in FOXO homozygous null flies

Due to the phenotypes observed using the *foxo* heterozygous flies, we were interested to see whether completely removing *foxo* had an effect on *fs(1)h* levels by using *foxo* homozygous null flies. In whole fly samples, we could confirm that *foxo* homozygous null flies had almost no *foxo* transcript level present (Figure 5-15A) and in these samples we could also show the *foxo* homozygous null flies had a significant upregulation in the transcript level of *fs(1)h* (Figure 5-15B). In the dissected fat body samples, we could again show there was no *foxo* present in the dissected fat body of the *foxo* homozygous null flies (Figure 5-15C), and we could also see a trend for an increase in *fs(1)h* transcript level in the *foxo* homozygous null flies (Figure 5-15D). After identifying increased *foxo* levels in *fs(1)h* knockdown flies, we could also show that *fs(1)h* transcript levels may be regulated by *foxo* expression levels. These data suggest that *fs(1)h* may be able to control FOXO protein abundance, but the transcription factor *foxo* may also regulate *fs(1)h* transcript levels.

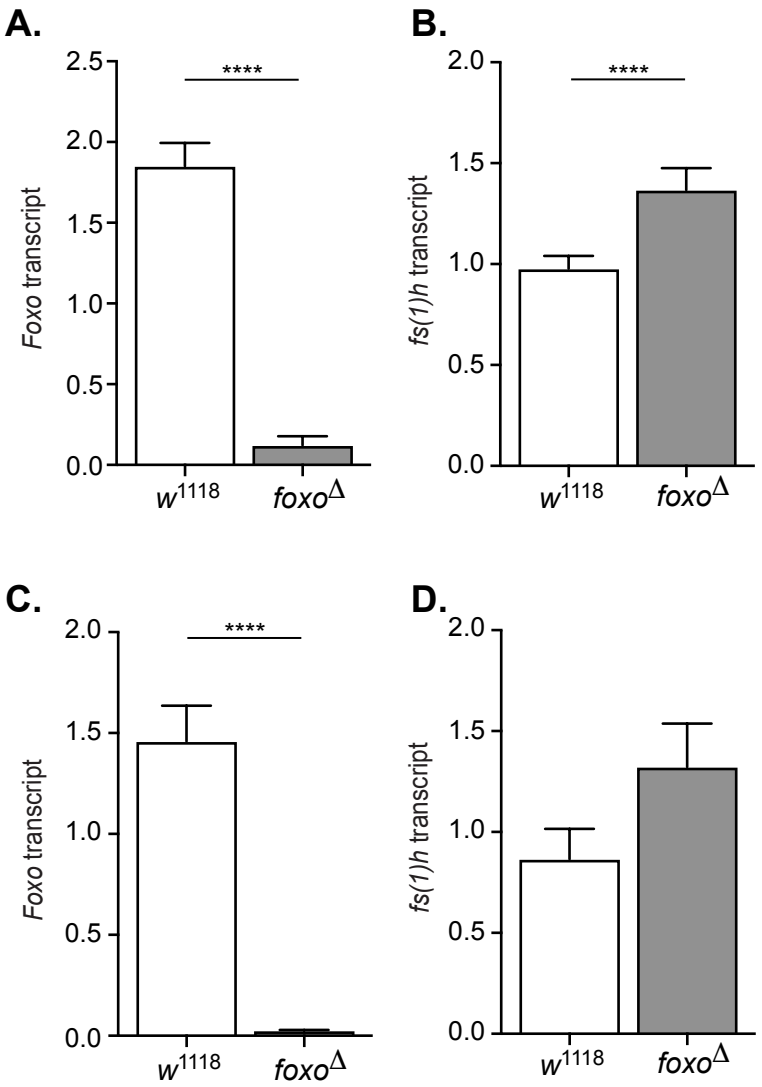


Figure 5-15: Confirming the loss of foxo and *fs(1)h* levels in foxo homozygous null flies
RT-qPCR analysis of 5-7 day old whole fly samples and dissected fat body samples of w^{1118} controls and *foxo* homozygous null flies ($foxo^{\Delta}$). All RT-qPCR data was normalised to the housekeeping gene, α-*tubulin*. Normalised transcript levels of **A**. *foxo* in whole fly samples, **B.** *fs(1)h* in whole body samples, **C**. *foxo* in dissected fat body samples and **D.** *fs(1)h* in dissected fat body samples. Unpaired t tests, ****p<0.0001, no stars indicate lack of statistical significance. Data represented as mean of n=10 biological repeats per genotype (3 flies per sample), error bars as SEM.

5.12. Chapter 5 overview

In this chapter, we have identified that the biological consequences of *fs(1)h* loss-of-function in the fat body are largely dependent on the elevated levels of the transcription factor *foxo.* We were able to show this by producing flies that were heterozygous for *foxo* in the *fs(1)h* fat body knockdown flies. Heterozygosity is the presense of two different alleles of a gene, in this case we were removing one copy of *foxo.* The heterozygous genotype with a functional *foxo* allele and a non-functional allele is usually expected to produce 50% of the standard activity of the gene (Wang et al., 2010). However, the phenotype of the heterozygote will depend on the haplosufficiency of the single functional allele, suggesting that the gene activity may be higher, or lower than the expected 50% activity (Deutschbauer, 2005).

Previously we showed *fs(1)h*, the sole BET protein in *Drosophila*, has critical functions in both immunity and metabolism. Remarkably, it appears that almost all these functions were due to a loss of AKT signalling and increased *foxo* levels. Removing a single copy of *foxo* completely rescued the reduced lifespan of *fs(1)h* knockdown flies, but did not reduce the survival when infected with *F. novicida*. *foxo* heterozygosity also restored the expression levels of uninfected AMPs, but did not rescue AMP expression following bacterial infection, while total Relish protein levels were normalised in both uninfected and infected individuals. The reduced starvation survival phenotype seen in *fs(1)h* fat body knockout flies was rescued along with the ability for flies heterozygous for *foxo* to utilise their triglyceride stores following 24-hour starvation. However, *foxo* heterozygosity does increase the baseline triglyceride level, due to the role *foxo* plays in regulating fat cell storage in steady state (DiAngelo and Birnbaum, 2009). The transcript levels of important lipid metabolism genes including *bmm, plin1* and *plin2*, were also restored in *foxo* heterozygous flies. Following *foxo* heterozygosity in the *fs(1)h* knockdown flies the reduced circulating sugar levels, of trehalose and glucose, were rescued. However, glycogen, the stored carbohydrate, remained low when flies were both heterozygous for *foxo* and lacking *fs(1)h* in the fat body. The impaired levels of activated AKT and S6 kinase found in *fs(1)h* knockdown flies were restored to control levels when a copy of *foxo* was also removed. A number of nutrient-responsive signals, including *upd2, Gbp2, CCHa2 and ImpL2* were dysregulated following the loss of *fs(1)h* in the fat body, however removing a copy of *foxo* rescued the transcriptional changes seen in these genes. *upd1,* a leptin-like factor, and *Gbp1,* an important gene in the regulation of the immune response (Koyama and Mirth, 2016) were not rescued following *foxo* heterozygosity. An increase in $FOXO^{GFP}$ protein level was identified following the loss of *fs(1)h* in the fat body, suggesting higher FOXO protein levels following the loss of

fs(1)h in the fat body. Furthermore, we identified first indications that flies with *fs(1)h* knocked down in the fat body may have ubiquitination dysfunction with changes in the proteins present and the quantity of some of the proteins. All these findings suggested there may be a defect in protein degradation upon ubiquitination when *fs(1)h* was removed from the fat body. Interestingly, this degradation defect seemed to be independent of *foxo* levels. This may suggest the elevated FOXO levels were due to a protein degradation fault developing in flies lacking *fs(1)h* in the fat body. Finally, our data showed that *foxo* homozygous null flies had an increased expression of *fs(1)h,* indicating there may be direct regulation between *fs(1)h* and *foxo* at a transcript level.

These data together suggest that *fs(1)h* knockdown may participate in a pathophysiological feedback loop via the dysregulation of FOXO and AKT activity, leading to altered expression of a number of endocrine signalling factors and in turn a reduction in systemic insulin activity. The reduction in insulin signalling and AKT activity may drive high FOXO activity, further disrupting the expression of insulin regulatory factors. Furthermore, these data could suggest that *fs(1)h* may directly affect *foxo,* which in turn leads to insulin pathway dysregulation. The use of *foxo* heterozygous flies may have reduced the increased level of FOXO and weakens this feedback loop back to a more physiologically normal homeostasis.

Chapter 6

Removing BET proteins from the human immune cell line, THP-1

6.1. Introduction

Epigenetic changes and histone modifications have been of great interest in recent years as abnormalities in the process can alter chromatin structure and ultimately lead to dysregulated gene expression and the development of disease (Kanherkar et al., 2014). In mammals, chromatin plays an important role in the regulation of inflammation by activating a cohort of inflammatory cytokines (Bernstein et al., 2007; Medzhitov and Horng, 2009; Smale, 2010). The process of acetylation, predominantly carried out by histone acetyltransferases (HATs), controls various inflammatory genes (Bayarsaihan, 2011). Furthermore, the promoters of several pro-inflammatory cytokines, including interleukin-1 (IL-1), IL-2, IL-8 and IL-12, are rapidly acetylated by CBP/p300 leading to transcriptional activation (Villagra et al., 2009). In contrast, the recruitment of histone deacetylase (HDACs) causes histone deacetylation and gene repression, HDACs also have the ability to regulate the transcription of various pro- and anti-inflammatory cytokines and transcription factors including *FOXP3*, *STATs* and *NF-κB* (Villagra et al., 2009). Acetylation of histones has many functional roles and the detection of these groups by epigenetic readers is extremely important for the control of transcriptional regulation.

Bromodomain-containing proteins (BCPs) are an important class of histone modification proteins that recognise acetylated lysine residues in histones to alter transcriptional regulation (Josling et al., 2012). The human genome encodes 46 BCPs, each of which contain from one to six bromodomains (BDs), creating a total of 61 unique human BCPs (Filippakopoulos et al., 2012). Structurally, BDs consist of a left-handed, four-helix bundle consisting of alphaZ, alphaA, alphaB and alphaC helices containing both hydrophilic and hydrophobic regions (Dhalluin et al., 1999). The architecture of the BD suggests it could act as an independent functional unit until it interacts with other proteins (Arrowsmith et al., 2012).

Gene expression is under the control of epigenetic mechanisms and several small-molecule inhibitors to target these processes have been developed (Tough et al., 2016). These tools have further revealed the importance of epigenetics in guiding cell fate during immune responses and have highlighted a new role as a treatment of inflammation and immune-mediated disease (Tough et al., 2016). Recent years have seen the development of therapeutic intervention for treating inflammation using a synthetic compound that targets the bromodomain and extraterminal domain (BET) family. The BET proteins, BRD2, BRD3, BRD4 and BRDT, are a family of epigenetic readers of acetylated lysine residues in histones, specifically at the amino-terminal tails (Schaefer, 2014). Acetylated histone tails are usually associated with transcriptionally active chromatin regions, and BET proteins play roles as effector

molecules, via the association and recruitment of various factors including transcription factors (Schaefer, 2014). Selective inhibitors of BET proteins are able to repress transcription by blocking their ability to recognise acetylated lysine residues (Filippakopoulos and Knapp, 2014). Small molecule BET inhibitors such as JQ1 (developed by the James Bradner Laboratory, Brigham and Women's Hospital) and I-BET (developed by GlaxoSmithKline) mimic acetyl moieties, occluding the bromodomains acetyllysine-binding pocket and displacing the BET protein from the chromatin (Zhang et al., 2016b).

JQ1 is a small molecule inhibitor that specifically targets BET proteins and acts by blocking the proteins ability to bind to chromatin (da Motta et al., 2017). In turn, this impairs the recruitment of numerous transcription factors to their target gene promoters (da Motta et al., 2017). Studies using JQ1 have shown its potential therapeutic role, with strong inhibitory effects on tumour growth and survival (Shao et al., 2014), cell cycle arrest and differentiation, along with the ability to alter hypoxia response, exerting an anti-tumour effect (da Motta et al., 2017). Furthermore, JQ1 inhibits mesenchymal stem cell (MSC) growth by influencing cellular processes including the cell cycle and signal transduction (Alghamdi et al., 2016).

I-BET, another small molecule BET inhibitor, binds selectively to the acetyl-recognising BET pocket with nanomolar affinity (Nicodeme et al., 2010). It leads to disruption of chromatin complexes that are responsible for the expression of inflammatory genes (Nicodeme et al., 2010). Initially, the first molecule, I-BET762 was used in an inflammatory disease model, showing that it potently inhibited expression of LPS inducible genes in macrophages *in vitro* and suppresses inflammation in a severe sepsis mouse model (Nicodeme et al., 2010). Another molecule, GSK1210151A (I-BET151) specifically inhibits the binding of bromodomain-containing proteins (BRD2, BRD3, BRD4 and BRD9) (Dawson et al., 2011) and exhibits potent selectivity over a range of protein types including PI3K-γ and G-protein coupled receptors (GPCRs) (Galdeano and Ciulli, 2016). Furthermore, it is an effective inhibitor of IL-6 production in LPS-stimulated peripheral blood mononuclear cells (PBMC) (Seal et al., 2012), osteoclastogenesis and inflammatory bone resorption (Park-Min et al., 2014). In rheumatoid arthritis (RA), I-BET151 suppresses TNF-α, IL-1β and TLR ligand induced expression of cytokines, chemokines and matrix metalloproteinases (MMPs) in the synovial fluid that normally contribute to the disease pathology (Klein et al., 2016). The development of inhibitors has revealed novel insights into the physiological and therapeutic functions of BET proteins and beneficial

effects of these inhibitors have been shown in a number of inflammatory conditions and cancer (Belkina and Denis, 2012).

In mammals, innate immune cells such as myeloid cells, including monocytes, macrophages and dendritic cells respond to environmental signs of pathogenic infection (Mogensen, 2009). Monocytes and macrophages are important cells of the immune response and they have three main roles; recognise foreign pathogens via pattern recognition receptors (PRRs) and phagocytose them, proliferate to increase the number of cells that are able to eliminate invading microorganisms and produce pro- and anti- inflammatory cytokines and chemokines to clear the site of infection and to keep the infection under control, respectively (Si-Tahar et al., 2009). THP-1 cells are a spontaneously immortalised human monocyte-like cell line, derived from the peripheral blood of an acute monocytic leukaemia patient (Tsuchiya et al., 1980). The THP-1 cell line is a simplified and reliable model to study monocyte and macrophage functions as well as macrophage differentiation (Chanput et al., 2014). Early studies demonstrate that THP-1 cells are similar to primary monocytes and macrophages both morphologically and functionally, including the presence of differentiation markers (Chanput et al., 2014). The cell line is used to study immune response while cells are in both a monocytic state and a macrophage-like state (Daigneault et al., 2010). THP-1 cells in the monocytic state can be differentiated into a macrophage-like cell using 1α, 25-dihydroxyvitamin D3 (vD3), phorbol-12-myristate-13-acetate (PMA) or macrophage colony-stimulating factor (M-CSF), usually in combination with IFN-γ (Aldo et al., 2013). A number of macrophage-like phenotypes have already been observed morphologically following differentiation, including adhering to culture plates and altering their morphology into flat, amoeboid cells (Tsuchiya et al., 1982). Following stimulation with LPS, THP-1 macrophages express *myeloid differentiation protein-2* (*MD-2*), *CD14* and *MyD88* genes, which are also required for LPS signalling *in vivo* (Chanput et al., 2014). Furthermore, genetically modifying THP-1 cells by using small interfering RNAs (siRNAs) to knockdown the expression of specific proteins is relatively simple in this cell line (Qin, 2012).

6.1.1. Objective and aims

The objective of this chapter is to investigate whether the effects on insulin signalling following the loss of *fs(1)h* in the fat body of *Drosophila,* can be reproduced in a human immune cell line.

The aims of this chapter are:

1. To investigate the effects on pAKT and FOXO in THP-1 cells following treatment with the bromodomain inhibitor, I-BET151.
2. To use siRNA to knockdown *Brd2*, *Brd3* and *Brd4* in THP-1 cells to investigate the roles they have individually on pAKT and FOXO levels.

6.2. Treating THP-1 cells with IBET-151 reduced AKT activity and increased FOXO levels

The human monocytic, THP-1, cell line was used to investigate if the effects we saw on the insulin signalling pathway when we knocked down *fs(1)h* in *Drosophila* fat body were conserved in human immune cells. Instead of genetically manipulating the bromodomain-containing protein, we used I-BET151 to inhibit the binding of the bromodomain to acetylated lysine residues in histones. THP-1 cells were treated for 24-hours with the bromodomain inhibitor, IBET-151. The treated cells showed a reduction in phosphorylated AKT protein levels (Figure 6-1A) and increased levels of FOXO3a protein (Figure 6-1B) when compared to THP-1 cells that had been treated with DMSO alone. Therefore, we could confirm a potential conserved role of BET proteins in the regulation of insulin signalling in immune cells from *Drosophila* to human.

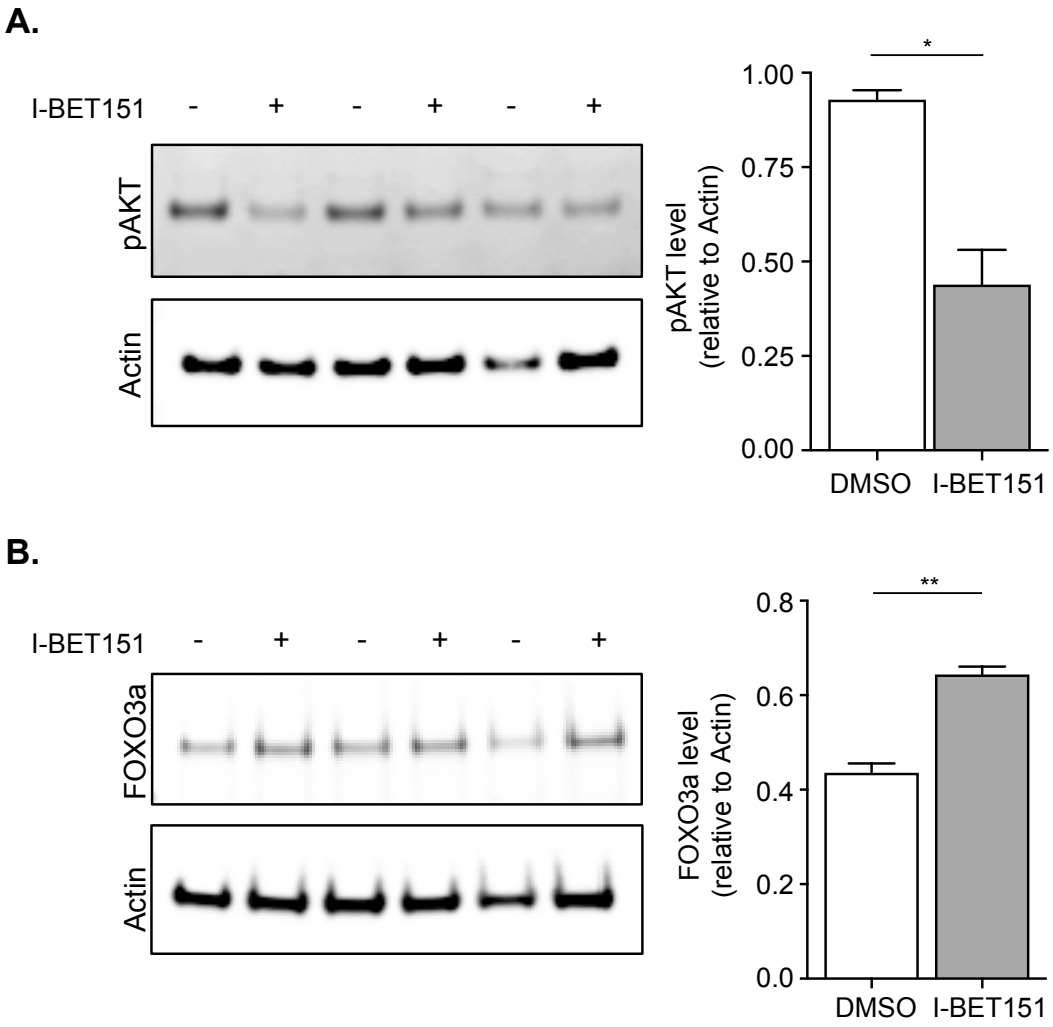


Figure 6-1: Treating THP-1 cells with I-BET151 showed changes in the insulin-signalling pathway

Western blot analysis of THP-1 cells following 24-hour treatment with the BET inhibitor, I-BET151, or DMSO as a control. **A.** Phosphorylated AKT (pAKT) protein level in I-BET151 treated cells (grey bars) and DMSO treated control cells (white bars). Values represented as intensity relative to Actin, data shown as mean + SEM, *p<0.05. **B.** FOXO3a protein level in I-BET151 treated cells (grey bars) and DMSO treated control cells (white bars). Values represented as intensity relative to Actin, data shown as mean + SEM, **p<0.01. +; presence of I-BET151, -; DMSO alone.

6.3. A knockdown of *Brd2* by siRNA showed no change in insulin signalling

The BRD2 protein is a member of the BET family, which associates with transcription complexes and acetylated chromatin during mitosis (Nakamura et al., 2006). Brd2 consist of four chromatin interacting domains that regulate gene expression, two N-terminal bromodomains, which recognise acetylated lysine residues, a protein-protein interaction domain at the C-terminal and an extraterminal (ET) domain that is highly conserved among BET proteins (Webby et al., 2009). The two bromodomains and the C-terminal domain are equally important in regulating transcription (Hnilicová et al., 2013). The first bromodomain (BD1) of BRD2 recognises H4K5ac or H4K8ac, whereas second bromodomain (BD2) interacts preferentially with H4K12ac or H4K5ac (Filippakopoulos et al., 2012; Huang et al., 2007; Umehara et al., 2010). Additionally, the bromodomains of BRD2 have a much higher affinity for tetraacetylated histone marks (H4K5ac, H4K8ac, H4K12ac and H4K16ac) rather than single acetylation marks (Hnilicová et al., 2013). BRD2 associates with E2 factor (E2F) family of transcription factors and helps to recruit TATA-binding protein (TBP) to promoter regions (Peng et al., 2007). Several experiments using mice have shown that the loss of Brd2 is detrimental. *Brd2*-deficient mice have developmental defects of the neural tube, abnormal brain structures and die during embryogenesis (Gyuris et al., 2009; Shang et al., 2009). Heterozygous *Brd2*-deficient mice are viable but develop spontaneous seizures and have a low number of inhibitory neurons in the brain (Velíšek et al., 2011). Furthermore, it was shown that mice with lower levels of *Brd2* are extremely obese, but protected from type 2 diabetes (Wang et al., 2009). In transgenic mice, where *Brd2* is constitutively expressed in B-cells, *Brd2* upregulates *cyclin* A, destabilises the cell cycle and leads to B-cell over proliferation and malignancy (Belkina et al., 2014; Blanton et al., 2008; Greenwald et al., 2004). In humans, BRD2 has been implicated in a number of diseases including cancer (Fu et al., 2015), chronic inflammation (Belkina et al., 2013) and epilepsy (Layouni et al., 2010; Pal et al., 2003). It has also been shown to be involved in the regulation of a number of signalling pathways including Wnt and Hedgehog (Engelke and Chinnaiyan, 2015; Tang et al., 2014).

We wanted to assay the role of BRD2 on the insulin-signalling pathway by looking specifically at the activation of the main kinase AKT and the downstream transcription factor FOXO3a in a human immune cell line. We used siRNA to knockdown *Brd2* in THP-1 cells and assayed the efficiency of the knockdown three days after siRNA treatment. We were able to show a significant reduction in BRD2

protein level compared to the control DMSO treated cells (Figure 6-2A). However, analysis of phosphorylated AKT (pAKT) in these cells showed no change in the protein level of activated AKT compared to the DMSO treated cells (Figure 6-2B). Furthermore, we could not detect a reduction in FOXO3a protein level following *Brd2* knockdown (Figure 6-2C) when compared to the cells treated with DMSO. In conclusion, we were able to show an efficient knockdown of *Brd2* using siRNA in THP-1 cells but we were unable to show an effect on insulin signalling in these cells, indicated by unaltered pAKT and FOXO3A protein levels.

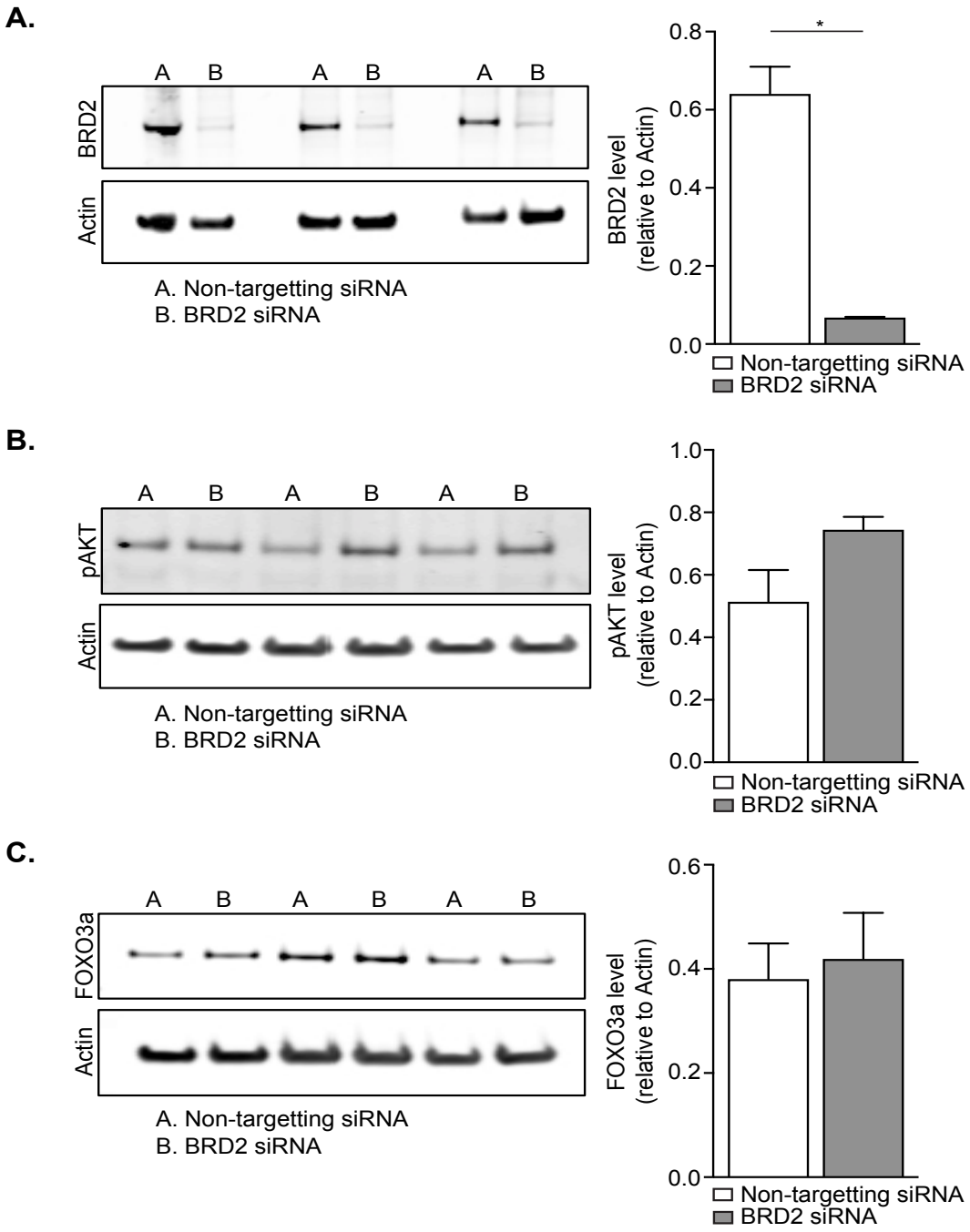


Figure 6-2: Using siRNA to knockdown *Brd2* did not show changes in the insulin-signalling pathway

Western blot analysis of THP-1 cells following *Brd2* siRNA or non-targeting siRNA as a control. **A.** Level of BRD2 protein following siRNA treatment, *Brd2* siRNA treated cells (grey bars) and non-targeting siRNA controls (white bars)**.** Values represented as intensity relative to Actin, data shown as mean + SEM,*p<0.05. **B.** Phosphorylated AKT protein level following siRNA treatment, *Brd2* siRNA treated cells (grey bars) and non-targeting siRNA controls (white bars)**.** Values represented as intensity relative to Actin, data shown as mean + SEM, no stars indicate lack of statistical significance. **C.** FOXO3a protein level following siRNA treatment, *Brd2* siRNA treated cells (grey bars) and non-targeting siRNA controls (white bars)**.** Values represented as intensity relative to Actin, data shown as mean + SEM, no stars indicate lack of statistical significance. A; non-targeting siRNA, B; *Brd2* siRNA.

6.4. *Brd3* knockdown in THP-1 cells showed no difference in insulin signalling

Bromodomain-containing protein 3 (BRD3) is a member of the BET protein family, and much like the other BET family members it associates with acetylated lysine residues in histones to regulate transcription (Lamonica et al., 2011). The first bromodomain (BD1) of BRD3 interacts with the GATA1 transcription factor and enhances the expression of GATA1-dependent genes (Gamsjaeger et al., 2011; Lamonica et al., 2011). BRD3 has also been shown to interact with acetylated lysine residues on the N-terminal of H2A, H2B, H3 and H4 (LeRoy et al., 2008). Additionally, *Brd3* promotes LPS-triggered IL-6 production by promoting the recruitment of CREB-binding protein (CBP) to the IL-6 promoter and enhancing the amount of acetylation of histone 3 close to the IL-6 promoter region (Ren et al., 2017). Knockdown of *Brd3* affects the expression and activation of signalling pathways involved in IL-6 expression, including *NF-κB* and mitogen-activated protein kinase (MAPK) pathways (Ren et al., 2016). Defects involving BRD3 are found in rare, aggressive and lethal carcinomas arising from midline organs including the intestine, bladder and pancreas (French, 2010). Furthermore, Shao et al (2016) found that an isoform of *Brd3*, known as Brd3 with Reprogramming activity (*Brd3R*) positively regulates mitosis during reprogramming. It is shown to upregulate a large set of mitotic genes during early reprogramming stages and associates with mitotic chromatin (Shao et al., 2016). Interestingly, depleting BRD3 levels has also been shown to slow cancer growth in mouse models of prostate cancer and medulloblastoma (Henssen et al., 2013; Wilting and Dannenberg, 2012).

We used siRNA to knockdown *Brd3* in THP-1 cells and found almost a complete loss of BRD3 protein in the three-day siRNA treated cells, compared to those treated with DMSO as a control (Figure 6-3A). However, there was no detectable change in the protein level of activated AKT (Figure 6-3B) or FOXO3a protein level (Figure 6-3C) when we compared the siRNA treated cells to the DMSO treated control THP-1 cells.

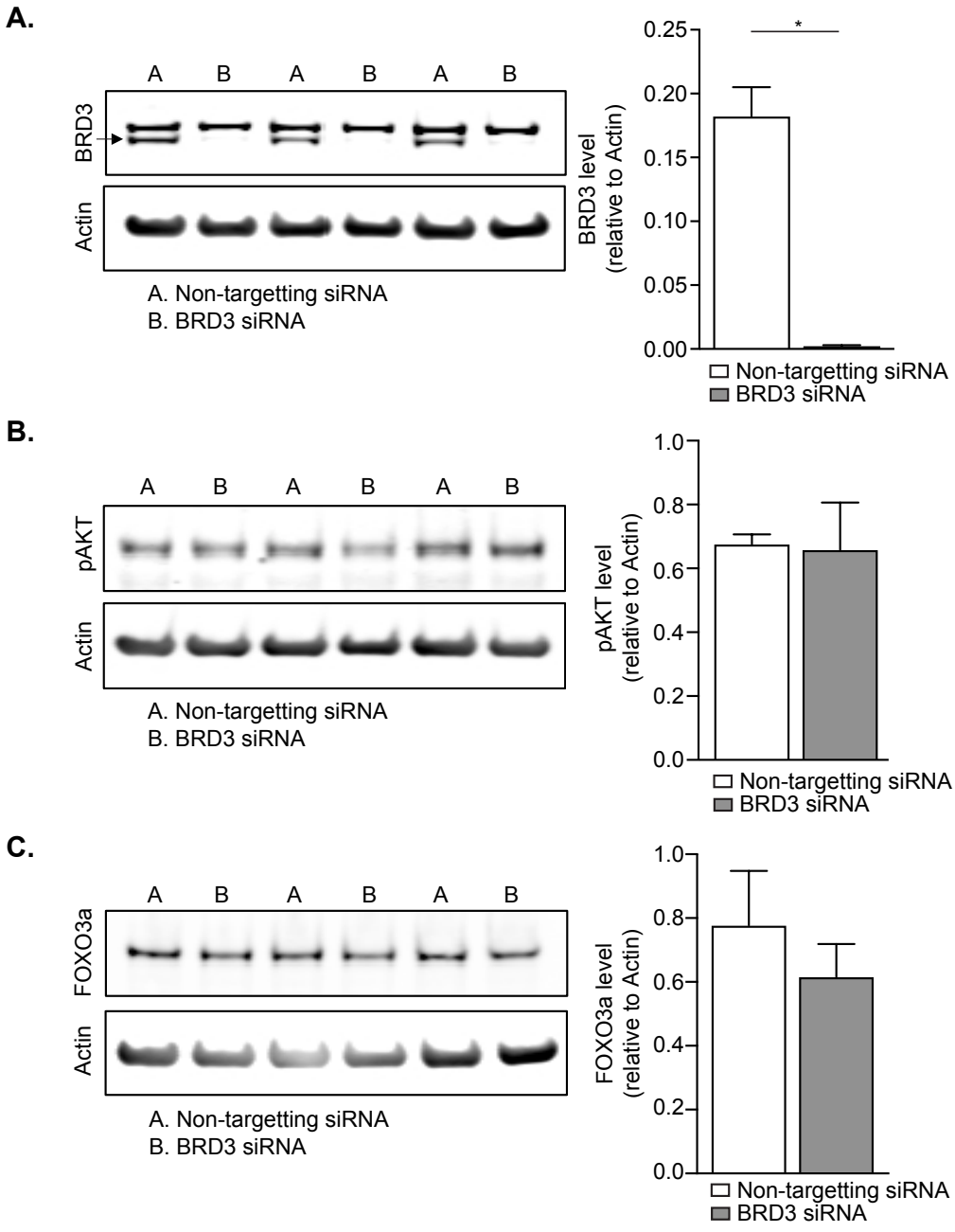


Figure 6-3: Using siRNA to knockdown *Brd3* did not show changes in the insulin-signalling pathway

Western blot analysis of THP-1 cells following *Brd3* siRNA or non-targeting siRNA as a control. **A.** Level of BRD3 protein following siRNA treatment, *Brd3* siRNA treated cells (grey bars) and non-targeting siRNA controls (white bars)**.** Values represented as intensity relative to Actin, data shown as mean + SEM.*p<0.05. **B.** Phosphorylated AKT protein level following siRNA treatment, *Brd3* siRNA treated cells (grey bars) and non-targeting siRNA controls (white bars)**.** Values represented as intensity relative to Actin, data shown as mean + SEM, no stars indicate lack of statistical significance. **C.** FOXO3a protein level following siRNA treatment, *Brd3* siRNA treated cells (grey bars) and non-targeting siRNA controls (white bars)**.** Values represented as intensity relative to Actin, data shown as mean + SEM, no stars indicate lack of statistical significance. A; non-targeting siRNA, B; *Brd3* siRNA.

6.5. Insulin signalling was not affected by *Brd4* knockdown using siRNA

Bromodomain-containing protein 4 (BRD4) is another member of the BET family, consisting of two tandem bromodomains and an extraterminal (ET) domain. BRD4 is an important chromatin binding protein that binds the acetylated lysine residues of histone H3 and H4 to activate transcription (Chiang, 2009). Additionally, BRD4 has a C-terminal domain that has been implicated in promoting gene transcription via the recruitment of the active positive transcription elongation factor (P-TEFb) and inducing the phosphorylation of RNA polymerase II (Devaiah et al., 2012; Hargreaves et al., 2009; Jang et al., 2005; Yang et al., 2005). BRD4 is essential for cellular growth, cell cycle control, DNA replication and gene rearrangement (Wu and Chiang, 2007), it has been suggested to function in 'transcriptional memory' to maintain the correct gene expression patterns during cell division (Bisgrove et al., 2007). TGF-β can induce the formation of an NF-κB-BRD4 complex and recruit BRD4 to epithelial-mesenchymal transition (EMT) transcription regulators (Tian et al., 2016). In a fibrosis mouse model, inhibiting BRD4 with small-molecule inhibitors can also reduce lung fibrosis following repetitive TGF-β challenge (Tian et al., 2016). BRD4 also seems to play a critical role in renal cell carcinoma (RCC). Inhibition of BRD4 activity results in supressed cellular proliferation and induces apoptosis in RCC (Wu et al., 2017). In addition, changes in BRD4 function can affect osteoblast differentiation at early stages as well as during mineralisation by regulating expression of skeleton or extracellular matrix-specific genes (Najafova et al., 2017). BRD4 is also involved in a number of stages and processes of the viral life cycle of Human Papillomavirus (HPV) and Human Immunodeficiency virus (HIV). The E2 protein targets the C-terminus of BRD4 to regulate HPV and HIV transcription (Bisgrove et al., 2007; Lee and Chiang, 2009; McKinney et al., 2016).

Again, we used siRNA to knockdown *Brd4* in THP-1 cells, three-days after siRNA treatment we analysed the BRD4 protein levels in the cells, but it was not as efficient as the siRNA for BRD2 or BRD3. We found only a slight reduction of BRD4 protein abundance following treatment with the siRNA compared to the DMSO treated cells (Figure 6-4A). There was also no change in the protein level of activated AKT (Figure 6-4B) or FOXO3A protein level (Figure 6-4C) when we compared the siRNA treated cells to the DMSO treated THP-1 cells.

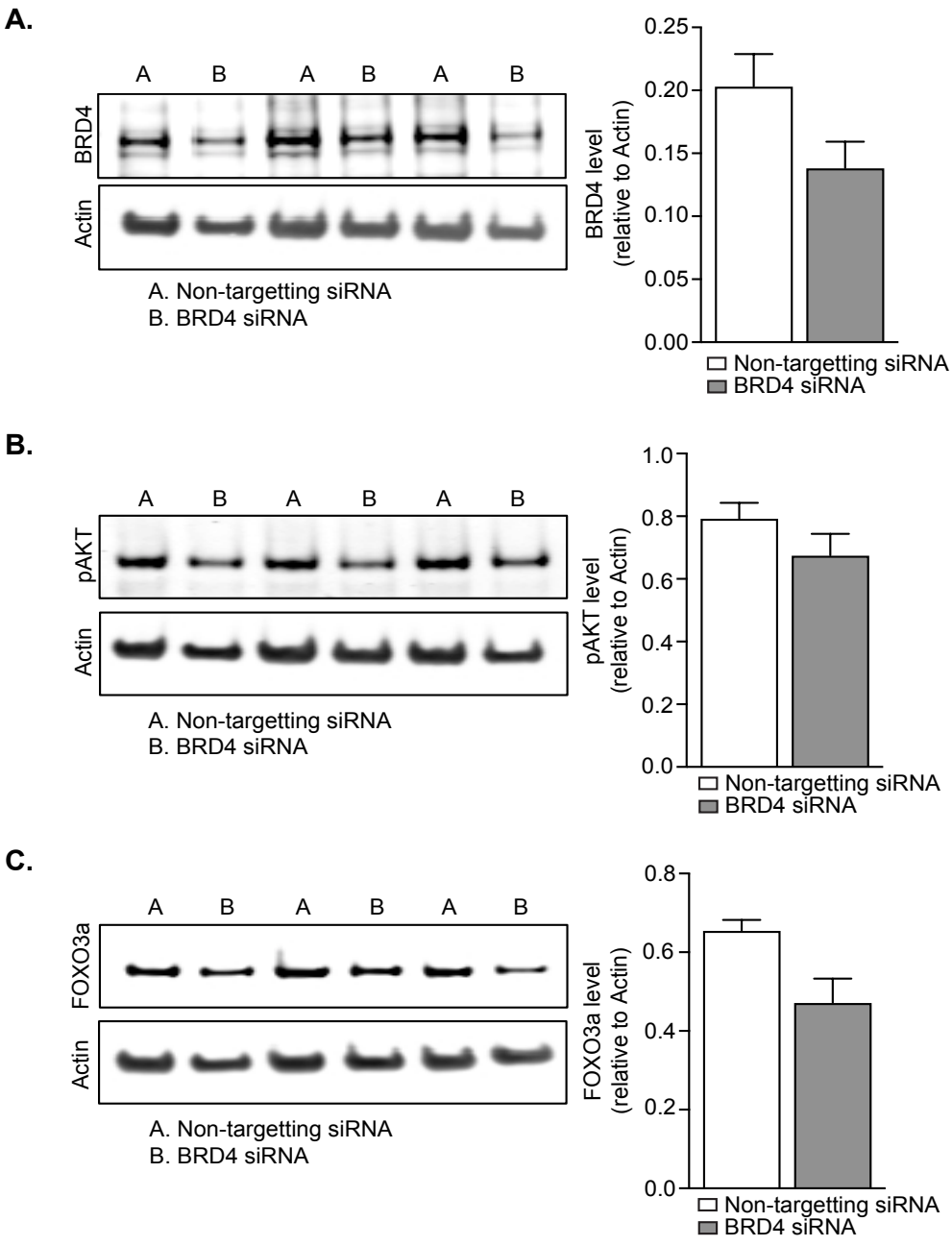


Figure 6-4: Using siRNA to knockdown *Brd4* showed no change in the insulin-signalling pathway

Western blot analysis of THP-1 cells following *Brd4* siRNA or non-targeting siRNA as a control. **A.** Level of BRD4 protein following siRNA treatment, *Brd4* siRNA treated cells (grey bars) and non-targeting siRNA controls (white bars)**.** Values represented as intensity relative to Actin, data shown as mean + SEM, no stars indicate lack of statistical significance. **B.** Phosphorylated AKT protein level following siRNA treatment, *Brd4* siRNA treated cells (grey bars) and non-targeting siRNA controls (white bars)**.** Values represented as intensity relative to Actin, data shown as mean + SEM, no stars indicate lack of statistical significance. **C.** FOXO3a protein level following siRNA treatment, *Brd4* siRNA treated cells (grey bars) and non-targeting siRNA controls (white bars)**.** Values represented as intensity relative to Actin, data shown as mean + SEM, no stars indicate lack of statistical significance. A; non-targeting siRNA, B; *Brd4* siRNA.

6.6. Chapter 6 overview

In this chapter, we have identified that the loss of BET proteins in the human immune cell line, THP-1, had similar effects on the insulin signalling pathway, as described previously when *fs(1)h*, the only BET protein in *Drosophila*, is removed from the fat body.

Bromodomain and extraterminal domain (BET) proteins play a crucial role in regulating gene transcription through an interaction between the bromodomains and acetylated lysine residues found predominantly in histone tails (Taniguchi, 2016). In recent years, the BET protein family have become a new therapeutic target in diseases including cancer, neurological disorders and inflammation (Padmanabhan et al., 2016). In *Drosophila*, we observed changes in the insulin signalling pathway when we knocked down *fs(1)h*, the sole *Drosophila* BET protein, in the fat body. We were able to detect a reduction in the activity of AKT, one of the main kinases of the insulin pathway and an increase in the level of FOXO following fat body knockdown of *fs(1)h*. The insulin-signalling pathway is highly conserved from *Drosophila* to humans, and we wanted to determine whether the results found in *Drosophila* were reproducible in a human immune cell line. THP-1 cells were used for these experiments; they are a widely used and a well characterised cell line used to investigate the function and regulation of monocytes and macrophages (Qin, 2012).

We found that when we treated the THP-1 human cell line with I-BET151, a small molecule inhibitor of the BET family of proteins, we were able to show a reduction in activated AKT levels (pAKT), similar to the reduction shown following knockdown of *fs(1)h* down in the fat body of *Drosophila.* We were also able to detect an increase in the protein level of FOXO in the THP-1 cells following 24-hour treatment with I-BET151. These data suggested there may be an important and conserved role for BET proteins in the regulation of the insulin-signalling pathway of immune cells. We also wanted to try and dissect these results further by using specific siRNA against the BET proteins (*Brd2*, *Brd3*, *Brd4*) and investigate whether one of these proteins was having a specific effect on the insulin-signalling pathway. Although the siRNA worked successfully for two out of the three BET protein knockdowns, we were unable to identify any changes in either activated AKT or FOXO3a levels. These data may suggest that the other BET proteins, which are still present after knockdown of one individual BET protein in the THP-1 cells, may be able to compensate for the loss of an individual BET proteins. It would be interesting to test the combined siRNA knockdown of *Brd2*, *3* and *4* in the THP-1 cell line. This may also suggest why the *fs(1)h* phenotype observed in *Drosophila* may be so strong, as there isn't another BET protein to recognise acetylated lysine residues and regulate transcription following

knockdown in the fat body. We were only able to test the conservation of this regulatory effect in the insulin-signalling pathway in one cell line. The regulation of the insulin pathway by BET proteins should be further tested on other human immune cells, but also in hepatocytes of the liver and adipocytes, as they share many properties with the fat body of *Drosophila.*

Chapter 7

Discussion

7.1. Introduction

The objective of this thesis was to investigate the role of bromodomain-containing proteins (BCPs) and jumonji domain-containing proteins (JDCPs) in the *Drosophila* immune cells, hemocytes and fat body, following bacterial infection. The survival screen resulted in the identification of *female sterile (1) homeotic (fs(1)h)* to be required in the fat body.

In this thesis, we showed for the first time that *fs(1)h, the sole Drosophila* member of the bromodomain and extraterminal (BET) protein family, was essential for both immunity and metabolism. *fs(1)h* had critical functions in the fat body to enable normal lifespan, restrain the expression of antimicrobial peptides (AMPs) and for normal utilisation of triglyceride stores. Knockdown of *fs(1)h* in the *Drosophila* fat body also led to significant reductions in baseline AKT phosphorylation and hypoglycemia (Figure 7-1). It appeared that almost all of these functions were due to effects on signalling via AKT and FOXO. Removing a single copy of *foxo*, along with the knockdown of *fs(1)h* in the fat body, was able to rescue the reduced lifespan, normalise AMP expression levels when uninfected, and restore their ability to utilise triglyceride stores following starvation. Phosphorylated AKT levels, S6 Kinase levels and circulating sugar levels were also rescued by *foxo* heterozygosity. Importantly, following bacterial infection, survival and AMP expression levels were not rescued by the loss of one copy of *foxo*, indicating that not all the *fs(1)h* phenotypes were attributed to FOXO hyperactivation. Interestingly, we also found the first indication for a potential regulation or interaction between *fs(1)h* and *foxo*.

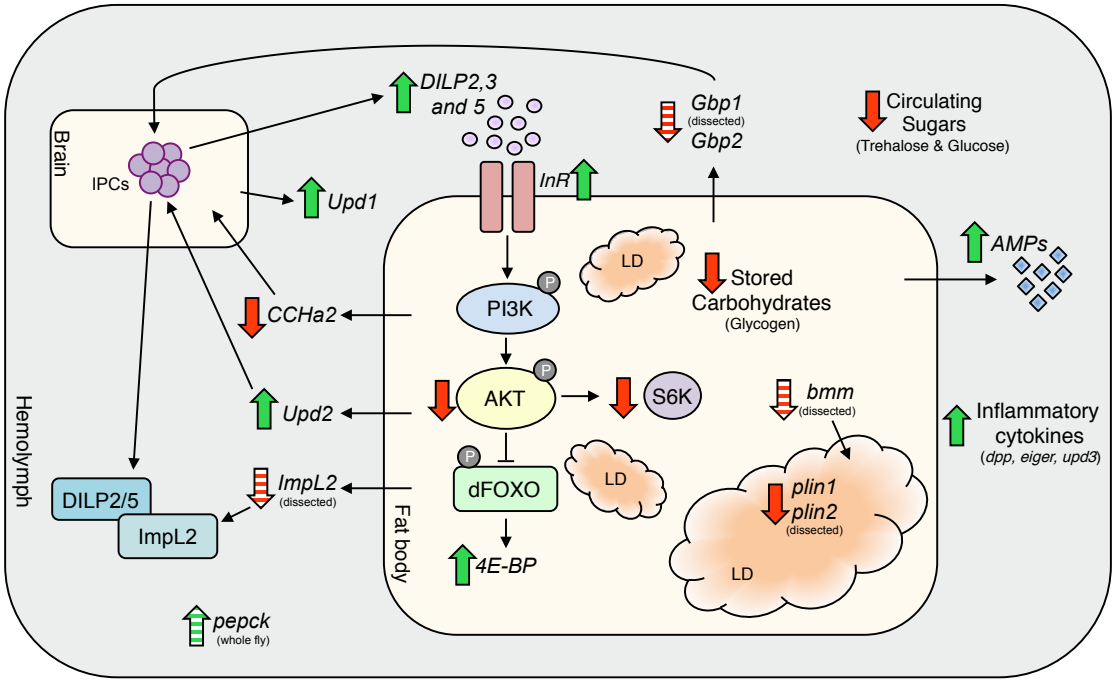


Figure 7-1: Summary of findings following *fs(1)h* knockdown in the *Drosophila* fat body
An overview of the data obtained in this thesis, showing changes that occurred in various signalling pathways, in various tissues following *fs(1)h* knockdown in the fat body. Green arrows indicate an increase in transcript or protein level, red arrows indicate a decrease in transcript or protein level. Striped arrows indicate differing results depending on whether whole fly or dissected fat body samples were analysed. LD; lipid droplet.

7.2. *fs(1)h* expression in the fat body is required for survival

Initially, we identified that *Drosophila* with a deficiency of *fs(1)h* in the fat body showed a reduction in survival following bacterial infection. Interestingly, *fs(1)h* knockdown in the fat body also led to a dramatically shortened lifespan under physiological conditions and during starvation. Ageing is accompanied by cellular damage, changes in repair and detoxification processes, and a shift in homeostatic balance, leading to dysregulation of various signalling pathways (Rubinstein and Kimchi, 2012). Human ageing and age-related disease is often associated with increases in inflammatory markers and insulin resistance (Chung et al., 2009; Lusis et al., 2008). In *Drosophila,* similarly to humans, ageing is associated with an increased expression of immunity-related genes (Landis et al., 2004; Pletcher et al., 2002) and changes in insulin signalling (Morris et al., 2012). *fs(1)h* knockdown in the fat body led to an over-expression of the antimicrobial peptides (AMPs) measured as well as severe changes in the insulin-signalling pathway, these are two pathological changes that may account for the decreased survival observed in the *fs(1)h* knockdown flies. Functional decline of organs and signalling pathways of individuals links both physiological ageing and the probability of death (Rera et al., 2012). The over-expression of AMPs

and the changes in the insulin-signalling pathway may have induced dysregulated organ function following *fs(1)h* knockdown in the fat body.

The intestinal epithelium is a regenerative, permeable membrane that interacts with normal microflora and pathogens to maintain homeostasis and elicit an immune response when needed (Ayyaz et al., 2015). In ageing mammals and *Drosophila*, structural and functional damage of the intestinal epithelium have been reported (Biteau et al., 2008; Kirkwood, 2004; Rera et al., 2011). This suggests maintaining gut integrity is an important indicator of organism health and viability (Rera et al., 2012). Furthermore, intestinal barrier dysfunction is correlated with lifespan of *Drosophila* and regardless of chronological age, gut barrier dysfunction has been reported as a predictor of death in flies dying of old age (Rera et al., 2012). Following knockdown of *fs(1)h* in the fat body, a decline in gut barrier function was observed in a small number of the flies, which may be another indication of their decreased viability. Although the main reason for the reduction in survival seems to be due to the reduction in AKT and the increase in FOXO levels following *fs(1)h* knockdown, there are also a number of other factors that may have contributed to the early death phenotype including toxic levels of antimicrobial peptides, hypoglycemia and dysregulation in endocrine signalling.

7.3. Expression of antimicrobial peptides and cytokines

Drosophila have a multi-layered immune system consisting of various defence mechanisms that can be used to help fight infection including physical barriers, along with a cellular and a humoral immune response. The cellular response is driven predominantly by hemocytes, to phagocytose and encapsulate intruding pathogens, and the humoral response, which produces the majority of the molecules to help kill the microbes (Brennan and Anderson, 2004; Hoffmann, 2003; Hultmark, 2003). Antimicrobial peptides (AMPs) are an important aspect of the humoral response and host defence in *Drosophila.* The AMPs are grouped on their biological targets, such as Gram-positive, Gram-negative bacteria or fungi (Imler and Bulet, 2005). These peptides are produced predominantly by the fat body, an organ analogous to adipose tissue and the liver in mammals, but can also be expressed by phagocytes of the fly known as hemocytes, as well as epithelial cells (Lemaitre and Hoffmann, 2007). In the initial survival screen, knocking down *fs(1)h* in hemocytes did not lead to a decreased survival following bacterial infection, unlike the dramatic reduction in survival observed following knockdown in the fat body. These data suggest *fs(1)h* expression may be important for the regulation of the humoral immune response in the fat body, but may be redundant for the cellular immune response in hemocytes. The upregulation of

AMPs may also indicate further that *fs(1)h* could be involved in the pathways prompting AMP expression in the fat body. Upon infection, two major immune signalling pathways, *Toll* and *Imd*, can be activated via members of the *NF-κB* family known as *Dif*, *Dorsal* and *Relish* (Tanji et al., 2007). Infection-induced AMP expression is due to strong signalling via NF-κB and MEF-2 combined with tissue identity signals from GATA transcription factors (Clark et al., 2013; Petersen et al., 1999; Senger et al., 2006). However, following *fs(1)h* knockdown in the fat body there was an increase in total Relish protein level, but there was no increase in activated Relish under physiological or infected conditions. These data suggest *fs(1)h* may affect total Relish levels, NF-κB signalling or their targets in the *Drosophila* fat body but not directly affect the activation of the *Imd* pathway.

It was previously described that under uninfected conditions, a subset of AMPs can be directly activated by the transcription factor, *foxo* depending on the metabolic state of the fly (Loch et al., 2017). However, *foxo* is not believed to be an important regulator of AMPs in the fat body during infection (Becker et al., 2010). These observations suggest there is cross-talk between the innate immune response and metabolism (Becker et al., 2010; Varma et al., 2014). The data suggested *fs(1)h* may be a key regulator of AKT and FOXO signalling, and could regulate AMP expression by controlling the level of *foxo.* In uninfected conditions, the elevated AMP expression following the loss of *fs(1)h* in the fat body seems to *foxo* dependent, as *foxo* heterozygosity is able to rescue the phenotype. Although total Relish protein was upregulated, the increase in AMPs did not seem to be due to an increase in *Imd* pathway activation. Interestingly, removing one copy of *foxo* during infection was unable to rescue the survival phenotype or the increased AMP expression in *fs(1)h* knockdown flies. These observations fit with the data showing that *foxo* may only control AMP expression under uninfected conditions (Becker et al., 2010). Furthermore under normal conditions, *fs(1)h* may play a role in constraining *foxo* activation to a low enough level that it does not activate AMPs. Infection-induced AMP expression was increased in *fs(1)h* knockdown flies independent of *foxo*, suggesting that under normal conditions *fs(1)h* may supress AMP loci. *fs(1)h* may be an endogenous negative regulator of AMP expression, in the presence or absence of infection.

Along with the role of AMPs in the innate immune response, they also help to maintain a steady state of microflora in the gut (Lehrer and Ganz, 1999), play an important role in tolerance to oxidative stress and are induced under non-infectious stress (Zhao et al., 2011). In the immune response of *Drosophila*, the NF-κB family member, *Dif* is dedicated to the antifungal defence elicited by fungi and Gram-positive

bacteria (Rutschmann et al., 2000). *Dif* mutant flies, much like *Toll* pathway mutants, are susceptible to fungal but not bacterial infections (Rutschmann et al., 2000). Whereas, *Imd* mutant flies are susceptible to bacterial but not fungal infections (Lemaitre et al., 1996), the ability to express *Drosomycin* is not affected in *Imd* mutants, but the expression of the other AMPs is impaired (Rutschmann et al., 2000). Loss of *fs(1)h* in the fat body resulted in a reduction of survival, but interestingly, these flies showed a hyperactivation of the immune response, rather than a down-regulation. In many organisms, a dysregulation of the immune response can result in severe pathologies and can contribute to tissue damage (Dionne and Schneider, 2008). Mutations in negative regulators of the *Imd* pathway disposes *Drosophila* to toxic levels of AMPs, resulting in reduced lifespan, neurodegeneration and locomotor defects (Kounatidis et al., 2017). The over-expression of AMPs in *fs(1)h* knockdown flies may be inducing tissue damage and a pathology that could explain the severely reduced lifespan. Additionally, during bacterial or viral infection, prolonged immune activation has been associated with metabolic dysregulation, mainly due to alterations insulin signalling (Dionne et al., 2006). In humans and *Drosophila*, excessive immune activation can drive metabolic disruption and the loss of metabolic stores (Dionne, 2014) and MEF2 in the fat body can act as a switch between both immune and metabolic functions (Clark et al., 2013). Furthermore, chronic activation of the immune response due to an over-expression of AMPs in neurons and glia of adult flies is sufficient to promote neurodegeneration (Cao et al., 2013; Kounatidis et al., 2017; Petersen et al., 2012; Sudmeier et al., 2015). Continuous upregulation of AMPs can be detrimental to host tissues (Bischoff et al., 2006; Zaidman-Rémy et al., 2006), following *fs(1)h* fat body knockdown the increased expression levels of AMPs may also be harmful to the flies.

In addition to the drastically elevated AMP expression, a number of cytokines were also elevated in unchallenged conditions following the loss of *fs(1)h* in the fat body. TGF-β family signals play roles in regulating tissue repair and inflammation in mammals (Li et al., 2006). *dpp*, a TGF-β family member in *Drosophila* is activated following wounding and is important for repressing the production of AMPs (Clark et al., 2011). This may explain the transcriptional increase in *dpp* expression observed following *fs(1)h* knockdown in the fat body as a rescue mechanism to reduce the increase in AMP expression during uninfected and infected conditions. In mammals, the TNF superfamily plays essential roles in regulating infection, inflammation and tissue homeostasis (Locksley et al., 2001). In *Drosophila, eiger,* is a TNF ligand that induces cell death by the activation of the JNK pathway (Igaki et al., 2002). Removing *fs(1)h* in the fat body of *Drosophila* caused an increase of *eiger*, which may be highly

expressed to try and help regulate and reduce the expression of AMPs and cytokines. The Jak/STAT pathway and one of its ligands, *upd3,* are involved in maintaining tissue homeostasis and tissue repair during times of infection and acute stress by controlling stem cell proliferation (Singh et al., 2007) and inhibiting apoptosis (Betz et al., 2008). *upd3* is produced as a pro-inflammatory cytokine by hemocytes in response to infection (Agaisse et al., 2003), but also in midgut epithelial cells, where it plays essential roles in gut infection and homeostasis (Buchon et al., 2009; Jiang et al., 2009; Osman et al., 2012). The production of *upd3* is induced in hemocytes following high fat diet and controls insulin sensitivity and lifespan in *Drosophila* (Woodcock et al., 2015). Over-activation of the Jak/STAT pathway can leads to persistent immune activation, inflammation and tissue damage (Shen-Orr et al., 2016). Therefore, increased *upd3* levels in *fs(1)h* knockdown flies could also play a part in causing the reduced survival phenotype.

7.4. Insulin signalling in the fat body

The insulin-signalling pathway is highly conserved in *Drosophila* and has many functional similarities to the mammalian insulin pathway (Papatheodorou et al., 2014). In response to sugars in the hemolymph, *Drosophila* insulin-like peptides (DILPs) bind to the insulin-like receptor (InR) and activate a signalling cascade via the phosphorylation of the main kinase, AKT. The activation of AKT controls the nuclear localisation of the transcription factor FOXO; following activation of AKT, FOXO remains inactive in the cytoplasm (Hay, 2011). AKT is also able to inhibit the TSC1/TSC2 complex, leading to the inhibition of Rheb and activation of TOR (Huang and Manning, 2009). Independently of DILPs and the InR, the TOR pathway can be regulated by amino acids, which are imported into cells via the amino acid transporter, Slimfast (Britton et al., 2002; Dann and Thomas, 2006). Insulin regulates growth of all tissues during the larval stage but in adults its effects are focused on lifespan, fecundity, metabolic homeostasis and resistance to stress (Broughton et al., 2005; Grönke et al., 2010). Mutations in single genes encoding insulin signalling components result in increased lifespan, reduced activity of the insulin pathway extends lifespan in the worm, fly and mouse (Kenyon, 2010), it also slows the ageing process (Hwangbo et al., 2004). Life expectancy in *Drosophila* is extended when the *InR* or *chico* are mutated. Conversely, the loss of *fs(1)h* in the fat body resulted in a reduction in AKT activity but also a decrease in survival. These observations suggested other phenotypes associated with *fs(1)h* knockdown in the fat body may be responsible for the reduction in lifespan, such as the increased AMP expression, their inability to utilise triglyceride stores or hypoglycemia. Ablating IPCs in *Drosophila*

larvae causes developmental delay, problems with growth and elevated carbohydrate levels (Rulifson et al., 2002). However, ablation of IPCs in the adult stage prolongs lifespan, increases storage of triglycerides and sugars, reduces fecundity and heightens resistance to starvation (Broughton et al., 2005).

Dietary restriction, where food intake is reduced without causing malnutrition, extends lifespan in many organisms from yeast to mammals (Anderson et al., 2003; Chippindale et al., 1993; Colman et al., 2009; Houthoofd et al., 2003; McCay et al., 1935; Partridge et al., 1987). In *Drosophila*, dietary restriction is applied by modifying the amount of yeast and diluting the nutrients in the food (Chapman and Partridge, 1996). Interestingly, the extension in lifespan is much greater in females than males, but also leads to reduced fecundity in *C. elegans*, *Drosophila* and rodents (Chapman and Partridge, 1996; Klass, 1977; Selesniemi et al., 2008). Given that a reduction in insulin signalling can increase *Drosophila* lifespan (Clancy et al., 2001; Tatar et al., 2001); this pathway may also help to mediate the response to dietary restriction. In mice, deletion of the insulin receptor in white adipose tissue results in lean, long-lived adults (Kenyon, 2005) and in *Drosophila*, the insulin receptor controls growth by regulating the size and number of cells and organs in response to nutrient availability (Brogiolo et al., 2001). The beneficial effects of reduced insulin signalling are evidently redundant in flies with *fs(1)h* knocked down in the fat body.

The loss of *fs(1)h* in the fat body also led to a reduction in lipolytic activity following starvation and a down-regulation of several lipid metabolism genes involved in energy mobilisation. Disruption of metabolic homeostasis often leads to the development of insulin resistance, a hallmark of type II diabetes (Morris et al., 2012). In mammals, hepatic deletion of AKT causes glucose intolerance and insulin resistance in hepatocytes (Yuan et al., 2012). However, *fs(1)h* knockdown in the fat body led to a reduction of activated AKT but the flies did not develop insulin resistance. In addition, disruption of Brd2 in mice causes severe obesity, hyperinsulinemia and hepatosteatosis (Wang et al., 2009). Furthermore, much like *fs(1)h* knockdown flies, mice lacking Brd2 experience elevated proinflammatory cytokines and reduced blood glucose (Wang et al., 2009). These data suggest *fs(1)h* in the *Drosophila* fat body and *Brd2* in the mouse may serve a similar function. Furthermore, S6 Kinase, a downstream effector of the PI3K signalling pathway (Dennis et al., 1999), which regulates ribosomal protein production and cell size, not cell number, in a cell-autonomous manner in *Drosophila* and in mammals (Montagne et al., 1999). *Drosophila* deficient in S6K have an extreme delay in development and a severe reduction in body size (Lambertsson, 1998), and flies dominant negative for S6K show lifespan extensions (Kapahi et al., 2004). Flies with *fs(1)h* reduction in the

fat body also showed a reduction in S6K, however, there were no morphological changes in the fat body tissue, suggesting the reduction in S6K was not causing a reduction in cell size or altering lifespan. The decrease in S6K activity may be caused by the reduction in AKT activity in *fs(1)h* knockdown flies, as its been previously described that AKT triggers S6K signalling via TOR and a reduction in pAKT levels can result in reduced S6K activity (Lizcano et al., 2003; Miron et al., 2003).

The forkhead box O (FOXO) family of transcription factors are key regulators of metabolism, ageing, stress resistance, independent of the immunoregulatory pathways. The reduction of pAKT in flies with *fs(1)h* knocked down in the fat body was accompanied by an increase in the transcript levels of *foxo* target genes, all which can be rescued by reducing the level of *foxo* in these flies. These findings suggest *fs(1)h* may be playing an important and direct role in regulating signalling via AKT and FOXO. Expression of *foxo* in early *Drosophila* development causes inhibition of larval growth and changes to feeding behaviour (Kramer et al., 2003). High levels of FOXO expression in early larval stages can cause developmental arrest and the inhibition of growth can be rescued when *foxo* expression is reduced (Kramer et al., 2003). In many organisms, FOXO is negatively regulated by AKT in response to insulin and insulin-like growth factors binding to insulin receptors. As mentioned previously, reducing insulin signalling and inducing the expression of FOXO in the fat body in the adult increases *Drosophila* lifespan (Hwangbo et al., 2004), although *fs(1)h* knockdown in the fat body displayed the opposite effect on survival. In mammals, hypoglycemia and a reduction in circulating insulin leads to FOXO1 localisation in the nucleus of hepatocytes, which drives gene expression to activate gluconeogenesis and lipid catabolism (Zhang et al., 2006). In line with these findings, knocking down *fs(1)h* in the fat body resulted in an increase in *Pepck* transcript, a major enzyme mediating gluconeogenesis. In non-infected flies, AMP genes are activated in response to nuclear FOXO activity when induced by starvation. AMP induction is lost in *foxo* null mutants, but enhanced when FOXO is over-expressed (Becker et al., 2010). Furthermore, over-expressing FOXO in flight muscles delays the accumulation of misfolded protein aggregates in muscle and non-autonomously in other tissues (Demontis and Perrimon, 2010). *foxo* also plays a functional role in response to stress, and a protective role during nutritional stress in larval development (Tettweiler et al., 2005). Interestingly, *foxo* upregulates transcript levels of *4E-BP,* a translational repression (also known as *Thor* in *Drosophila*), under conditions of low insulin (Jünger et al., 2003). FOXO and its target *4E-BP* also regulate the expression of various autophagy genes and protein homeostasis. In *Drosophila, foxo* mutants show proteostatic dysfunction and both *foxo* and *4E-BP* delay muscle function and extend

lifespan (Demontis and Perrimon, 2010). Knocking down *fs(1)h* in the fat body suggests that disturbed expression of endocrine factors resulted in low systemic insulin activity, which in turn drove high FOXO activity, further disrupting the expression of insulin and energy mobilisation regulatory factors.

7.5. Utilisation of triglycerides and sugar regulation

In humans, obesity is associated with many health implication including type II diabetes, cardiovascular disease and cancer (Lee and Mattson, 2014). However, under-storage, or an inability to utilise fat stores are also problematic particularly during periods of food deprivation. The main functions of lipids are to store energy in the form of triglycerides, compose cellular membranes and serve as precursors for a range of hormones (Liu and Huang, 2013). In *Drosophila,* storage lipids in the form of triglycerides are stored in lipid droplets in the fat body. Signalling pathways and major metabolic enzymes involved in lipid metabolism are conserved between *Drosophila* and mammals, including a number of genes that regulate lipid uptake, storage and mobilisation. When energy is required, lipids undergo lipolysis by lipases and hydrolases, releasing fatty acids, as energy, into the hemolymph (Moghadam et al., 2015). The mobilisation of lipids is regulated by hormonal signals and carried out by lipases produced by the fat body. The metabolic pathways and the lipases play essential roles in maintaining lipid homeostasis (Moghadam et al., 2015). In *Drosophila*, there are four main lipases that operate at the surface of lipids to breakdown triglycerides (TAG) into free fatty acids and glycerol (Pistillo et al., 1998). *Hormone-sensitive lipase* (*Hsl*) and *brummer* (*bmm*, homolog of human adipose triglyceride lipase; ATGL) are involved in the mobilisation of TAG. Chronic over-expression of *bmm* or lack of food depletes fat stores *in vivo*, and like ATGL knockout mice, *bmm* mutants have impairments in fat mobilisation and have increased TAG storage (Grönke et al., 2007). *Drosophila Hsl* mutants are partially defective in fat mobilisation, especially during starvation. Furthermore, *Hsl*;*bmm* double mutants are extremely obese (Grönke et al., 2005). The recruitment of Hsl or bmm to lipid droplets can be either supported or blocked by the perilipins (*plin1* and *plin2*) that are expressed primarily by the fat body and localise to the surface of lipid droplets (Bi et al., 2012). Interestingly, *plin1* and *plin2* mutant mice are lean, as they are thought to store less fat (Tansey et al., 2001), whereas *Drosophila plin1* mutants have large lipid droplets and an inability to mobilise lipids during starvation (Bi et al., 2012). *fs(1)h* knockdown in the fat body led to a reduction, but not an elimination, of *bmm, plin1* and p*lin2,* which may have contributed to the defect in energy mobilisation and the reduction in survival following starvation.

To maintain metabolic homeostasis, organisms have to coordinate the mobilisation of lipids, proteins and sugars to ensure energy levels across all tissues are adequate. Glucose metabolism in mammals is regulated predominantly by insulin signalling, and key metabolic pathways have been shown to be evolutionarily conserved from *Drosophila* to humans (Owusu-Ansah and Perrimon, 2014). In *Drosophila*, much like in mammals, circulating sugars are regulated by two endocrine hormones, insulin-like peptides (ILPs) and a glucagon-like peptide, Adipokinetic hormone (Akh) (Yasugi et al., 2017). Dietary sugars quickly change the levels of circulating sugars (Ugrankar et al., 2015), elevated glucose levels initiate insulin signalling, while starvation can cause glycogen breakdown and lipid mobilisation (Hietakangas and Cohen, 2009; Teleman, 2009). Trehalose is the main circulating sugar in invertebrates (Elbein et al., 2003; Shukla et al., 2015). In *Drosophila*, trehalose is produced from glucose in the fat body and degraded to glucose by trehalase (Yasugi et al., 2017). Much like in humans, an imbalanced diet can disrupt metabolic homeostasis in adult *Drosophila* and promote insulin resistance. *Drosophila* fed a high-fat diet are characterised by increased triglycerides, inflammation, metabolic imbalance (as hyperglycemia and insulin insensitivity) and a reduction in lifespan (Woodcock et al., 2015). Additionally, a high-sugar diet also elicits hyperglycemia and insulin resistant phenotypes (Musselman et al., 2011). However, the loss of *fs(1)h* in the fat body did not cause insulin resistance or obesity. Conversely, hypoglycemia and an increase in DILP expression are suggestive of increased insulin signalling (Flatt et al., 2008b). Genetic alterations in DILPs or Akh changes levels of trehalose and glucose circulating in the hemolymph and flies lacking Akh-producing neurons display a drop in trehalose levels (Lee and Park, 2004). Removing *fs(1)h* from the *Drosophila* fat body resulted in increased DILP secretion from the IPCs in the brain and hypoglycemia. The disturbance of endocrine factors regulating nutrient sensing and DILPs may account for the changes in insulin signalling and hypoglycemia shown in *fs(1)h* knockdown flies. Similarly to fat body knockdown of *fs(1)h* in *Drosophila*, mice deficient in Sirtuin-6 (SIRT6), a NAD-dependent protein deacetylase, which deacetylates lysines in histones and non-histone proteins (Ramakrishnan et al., 2014), have low insulin levels, are severely hypoglycemic, age prematurely and leads to the activation of NF-κB signalling (Xiao et al., 2010). Furthermore, Brd2 knockout mice, much like *fs(1)h* knockdown flies are shown to have lowered blood glucose levels (Wang et al., 2009).

The *Drosophila* genome encodes eight *Drosophila* insulin-like peptides (DILPs), which are expressed by various organs including the IPCs in the brain, fat body, midgut and ventral nerve cord (VNC) (Kannan and Fridell, 2013). DILP2, 3 and

5, are secreted from the IPCs in the brain, which are homologous to pancreatic beta cells in vertebrates and are shown to be nutrient responsive (Shim et al., 2013). Increasing the level of circulating DILPs systemically increases growth and metabolism (Lee et al., 2008b). *DILP2* is a potent growth regulator and is important in regulating the longevity of the adult fly (Morris et al., 2012), while *DILP3* and *5* are thought to control growth and development (Grönke et al., 2010). Ablating IPCs in *Drosophila* larvae result in a hyperglycemic phenotype that persists into adulthood (Broughton et al., 2008). Adult *Drosophila* with ablated IPCs in the brain are shown to be hyperglycemic and have impaired glucose tolerance, however they remain insulin sensitive (Haselton et al., 2010). This ablation of the IPCs would suggest a loss of DILP2, 3 and 5 secretions from the brain. Conversely, *fs(1)h* knockdown flies exhibited the opposite of these observations, with a hypoglycemic phenotype and a transcriptional increase in *DILP2*, *3* and *5*. These data suggest *fs(1)h* may play a role in regulating DILP secretion and sugar levels. The fat body-derived peptide, DILP6, acts indirectly, by stimulating the release of DILPs from other tissues. DILP6 has been shown to activate insulin signalling in oenocytes during starvation and promotes metabolic alterations when needed (Chatterjee et al., 2014). *DILP6* upregulation in the fat body during fasting or over-expression of *foxo* can repress secretion of DILP2 from IPCs in the brain (Bai et al., 2012). Over-expression of *DILP6* in the fat body causes traits associated with decreased insulin signalling, including lifespan extension and increased triglyceride levels (Bai et al., 2012). It is unknown whether *upd2* and *DILP6* work in parallel or in concert to regulate DILP secretion from IPCs (Owusu-Ansah and Perrimon, 2014). Interestingly, DILP6 is also able to repress the secretion of DILP2 from IPCs in the brain (Bai et al., 2012) however, knocking down *fs(1)h* in the fat body had little effect on the transcript levels of *DILP6*.

In mammals, phosphoenolpyruvate carboxykinase (*Pepck*) is a key enzyme for gluconeogenesis and glyceroneogenesis (Okamura et al., 2007), it has also been shown to play a key role in lipid homeostasis in white adipose tissue (Reshef et al., 2003). Mice expressing *Pepck* in adipose tissue displayed larger adipocytes and fat mass (Franckhauser et al., 2002). Conversely, mice with *Pepck* knockdown in adipose tissue exhibited lower triglyceride levels under both fed and fasted conditions (Olswang et al., 2002). In *Drosophila, Pepck* also plays an important role in gluconeogenesis and glyceroneogenesis and *ATF-2* of the p38 pathway has been shown to regulate *Pepck* transcript level. Furthermore, the expression of *Pepck* is reduced following *ATF-2* knockdown (Okamura et al., 2007). *fs(1)h* knockdown flies are shown to have increased *Pepck* transcript level, which may be associated with generating circulating sugars, to try and reduce their hypoglycemia. There may also

be an increase in ATF-2 and a stress-response via the p38 pathway, which is upregulating *Pepck* levels. This was not investigated following *fs(1)h* knockdown in the fat body. However, *Pepck* is also upregulated upon starvation (Zinke et al., 1999), which would fit with *fs(1)h* knockdown flies dramatically responding to starvation, and being unable to utilise triglyceride stores.

7.6. Nutrient sensing

Hormonal signalling ensures organ-to-organ communication, and failure to regulate this cross-talk in humans can lead to diseases including obesity and diabetes. Insulin-like growth factors (IGFs) and insulin in vertebrates, and insulin-like peptides (ILPs) in insects help to coordinate nutritional state and the control of growth (Okamoto and Yamanaka, 2015). In *Drosophila,* endocrine mechanisms and signals regulate carbohydrate and lipid metabolism and organs including the fat body, gut and brain can functions as nutrient sensors to coordinate growth depending on the nutritional conditions.

unpaired 1 (*upd1*), an endogenous ligand of the Jak/STAT pathway, is a mammalian leptin homolog expressed in the *Drosophila* brain, unlike *upd2*, which is also thought to be a human leptin homolog, expressed in the *Drosophila* fat body (Beshel et al., 2017). *upd1* acts through the *dome* receptor found on neuropeptide F (npf)-positive neurons, the upd1-dome signalling suppresses npf activity to regulate food-related behaviour including food intake, cues and weight (Beshel et al., 2017). Manipulation of *upd1* function in neurons impacts on feeding behaviour and responsiveness, leading ultimately to weight gain (Beshel et al., 2017). A decline in nutrient availability may contribute to a decrease in *upd1* expression and secretion (Beshel et al., 2017). Interestingly, *fs(1)h* fat body knockdown flies showed increased *upd1* transcript levels, suggesting that they were not exhibiting a reduction in nutrient availability. Although, *upd1* and *upd2* both activate the Jak/STAT pathway, they have different binding affinities for their receptor *dome,* suggesting different roles in *Drosophila* nutrient sensing. As mentioned previously, unpaired 2 (*upd2*), a type I cytokine and a ligand of the Jak/STAT pathway, was found to be induced in the fat body during the fed state in *Drosophila* (Rajan and Perrimon, 2012). *upd2* activates Jak/STAT in GABAergic neurons relieving the inhibitory effect of IPCs, leading to the secretion of DILPs into the hemolymph to promote growth and energy storage (Rajan and Perrimon, 2012). This induction of *upd2* in the fat body is also associated with the release of DILPs from IPCs in the brain (Rajan and Perrimon, 2012). Following *fs(1)h* knockdown in the fat body, transcript levels of *upd2* and *DILP2*, *3* and *5* were upregulated. The induction of DILP expression may be upregulated by the increased

upd2 expression in *fs(1)h* knockdown flies to try and rescue the dysregulated insulin-signalling pathway, independent of nutritional status. Much like in *Drosophila*, vertebrate leptin is secreted from adipose tissues in mammals during nutritional surplus (Zhang et al., 1994). Human leptin can function as a ligand to the dome receptor of the Jak/STAT pathway and has been shown to rescue *upd2* mutant phenotypes, suggesting *upd2* is a homolog of leptin (Rajan and Perrimon, 2012). Knocking down *upd2*, but not *upd1* or *upd3*, in the larval fat body resulted in smaller flies, suggesting *upd2* alone plays a fat body specific role in regulating systemic growth depending on nutrient availability (Rajan and Perrimon, 2012). Much like the knockdown, *upd2* null mutants are also smaller in size, have reduced triglyceride levels and accumulate lipid droplets in oenocytes. Over-expression of *upd2* can suppress lipid storage breakdown under conditions of starvation, suggesting it signals a fed condition even when flies are lacking nutrient (Rajan and Perrimon, 2012). In *fs(1)h* knockdown flies, there may be a feedback loop to increase *upd2* levels, as a stress signal, to try and induce DILP secretion from the IPCs in the brain to increase DILP binding to the InR and increase the amount AKT activation, in turn reduce FOXO level. The idea of a feedback loop was also supported by the loss of *fs(1)h* in the fat body leading to an increased expression of *InR,* which may be induced by FOXO in order to rescue the diminished AKT activity. Furthermore, the increased *upd2* transcript level following *fs(1)h* knockdown in the fat body may also be influencing the inability to utilise triglyceride stores, particularly following starvation.

Growth-blocking peptide (*Gbp*) was identified in butterflies and moths as a growth regulator, low levels of Gbp enhance growth, whereas high concentrations suppress it (Hayakawa and Noguchi, 1998; Matsumoto et al., 2003). In insects, *Gbp* is a potent cytokine that regulates stress-induced AMP expression, which does not require *Toll* or *Imd* related genes, and an over-expression of *Gbp* in *Drosophila* leads to elevated levels of AMPs (Tsuzuki et al., 2012). The larval fat body of *Drosophila* secretes epidermal growth factor-like peptides known as Gbps (Koyama and Mirth, 2016). *Gbp1* and *Gpb2* are produced by the fat body, secreted into the hemolymph and are able to stimulate DILP2 and DILP5 secretion, either directly or indirectly from IPCs in the brain (Koyama and Mirth, 2016), the stimulation of DILP secretion can lead to an increase in insulin signalling. Additionally, *Gbp1* and *Gbp2* transcript levels are sensitive to the protein content in the *Drosophila* diet and TOR signalling in the larval fat body, suggesting they work downstream of TOR (Koyama and Mirth, 2016). Reducing *Gbp1* and *Gbp2* expression in the *Drosophila* fat body leads to smaller body size due to reduced growth rate (Koyama and Mirth, 2016). Data suggests *Gbp1* and *Gbp2* do not play equal roles in regulating DILP secretion as the accumulation of

DILP2 was not enhanced by *Gbp2* knockdown, but increased when both *Gbp1* and *Gbp2* were reduced in the fat body (Koyama and Mirth, 2016). Furthermore, *Gbp1* regulates immune response and its expression is sensitive to starvation and TOR signalling in *Drosophila* (Fujikawa et al., 2009; Koyama and Mirth, 2016; Nelson et al., 2005). Interestingly, *fs(1)h* knockdown flies had reduced levels of *Gbp1* and *Gbp2*, but increased levels of AMPs suggesting *foxo* levels may be impacting on the AMP expression directly. Moreover, the transcript levels *Gbp1*, which regulates immune response, could not be rescued by *foxo* heterozygosity. These data further suggest *fs(1)h* knockdown may play two distinct roles in the fat body (Figure 7-2); a metabolic role that is *foxo*-dependent that could be rescued by removing one copy of *foxo,* and an immune role, predominantly following bacterial infection, that is *foxo*-independent, and cannot be rescued by reducing foxo levels in flies with *fs(1)h* knocked down in the fat body.

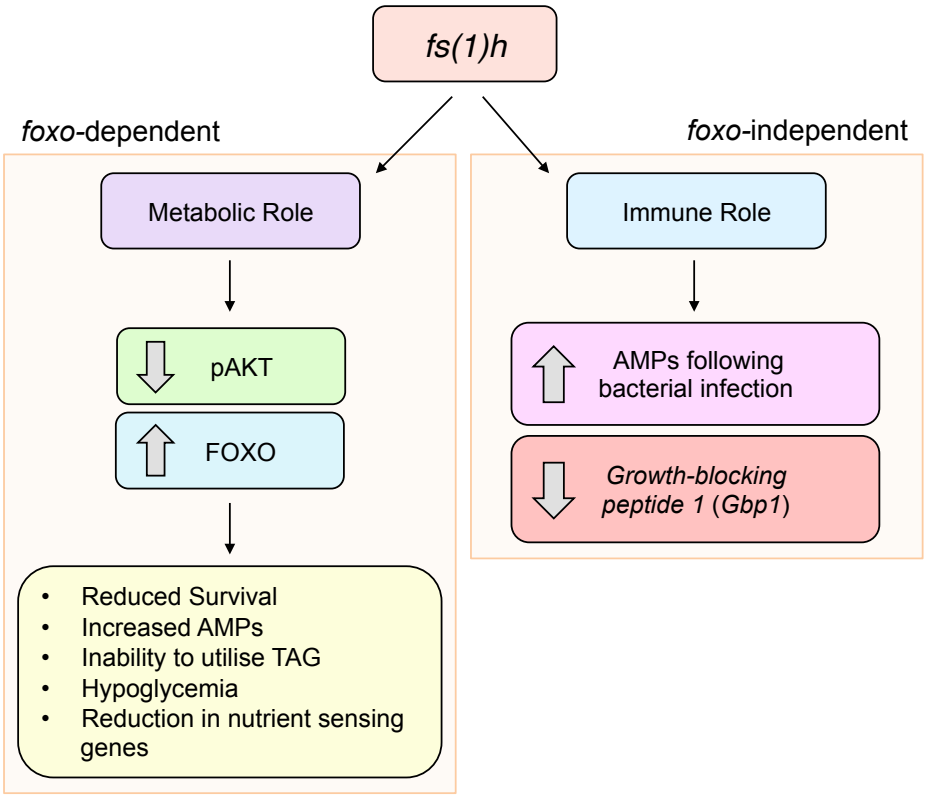


Figure 7-2: The distinct metabolic and immune roles of *fs(1)h* in the *Drosophila* fat body
fs(1)h seems to play two distinct roles in the *Drosophila* fat body. Left: A metabolic role that was dependent on *foxo* and many of the phenotypes observed following *fs(1)h* knockdown could be rescued by a reduction in *foxo* levels. Right: An immune role that is *foxo*-independent and a reduction in *foxo* levels in *fs(1)h* knockdown flies could not rescue some of the observed phenotypes.

CCHamide-2 (*CCHa2*), a peripheral tissue derived peptide hormone, is thought to be a nutrition-dependent regulator of DILPs in *Drosophila* larvae (Sano et al., 2015). *CCHa2* and its receptor, *CCHa2-R*, a G-protein coupled receptor (GPCR)

found in the brain, form a direct link between peripheral tissues and the brain. CCHa2 has been identified as a sugar- and lipid-sensitive humoral signal secreted from the fat body, similarly to *upd2* (Sano et al., 2015). In larvae, genetic disruption of *CCHa2* or *CCHa2-R* causes expression of *DILP5* and the release of DILP2 and 5 to be severely reduced (Sano et al., 2015). Interestingly, when *CCHa2* is expressed in the fat body of *CCHa2* mutants the level of *DILP5* is restored in the brain. Transcriptional levels of *CCHa2* are altered in response to nutritional levels, particularly glucose (Sano et al., 2015). In the *fs(1)h* knockdown flies, both *CCHa2* levels and glucose levels were reduced suggesting *fs(1)h* may also play a role in fat body-derived nutrient sensing and regulation. However, other studies suggest *CCHa2* is not regulating DILP secretion in the brain via the larval fat body, as no expression of *CCHa2* was identified in the fat body (Ren et al., 2015). It may be produced in the gut (Li et al., 2013; Ren et al., 2015; Veenstra and Ida, 2014) and signals to the *CCHa2-R* in the brain to plays important roles in controlling developmental timing (Ren et al., 2015), feeding behaviour (Li et al., 2013; Ren et al., 2015) and as a major regulatory peptide in the midgut (Veenstra and Ida, 2014). Possible signals from the gut lumen may communicate information, potentially regarding nutritional status and availability, to other organs in the fly by releasing *CCHa2* into the hemolymph (Li et al., 2013). Both larval and adult *CCHa2* mutants show a delayed development, significant reduction in feeding, decreased locomotion and a severe down-regulation of *DILP2* and *3* mRNA levels (Ren et al., 2015). It is uncertain whether glucose sensing takes place in *CCHa2*-expressing gut cells or in the fat body. Although *fs(1)h* knockdown in the fat body showed a significant reduction in *CCHa2* transcript, they did not seem to show any developmental delay or a reduction of *DILP2* or *3*. *fs(1)h* knockdown flies should be investigated further to identify whether the reduction in *CCHa2* is affecting their feeding behaviour.

The secreted protein, Ecdysone-inducible gene L2 (ImpL2), is induced by the molting hormone 20-hydroxyecdysone (20E, ecdysone) and is a neural/ectodermal development factor in *Drosophila* (Honegger et al., 2008). Genetic disruption of *ImpL2* results in embryonic lethality (Garbe et al., 1993). Furthermore, ImpL2 is an insulin-binding antagonist able to bind to *DILP2* and *DILP5* in *Drosophila* adults and is involved in a feedback circuit with *DILP2, 3* and *5* (Alic et al., 2011). Removing *DILP2, 3* and *5* leads to a down-regulation of *ImpL2* transcript (Alic et al., 2011). Conversely, increasing the expression of *ImpL2* results in phenotypic changes consistent with the down-regulation of insulin signalling, including increased lifespan (Alic et al., 2011). Forced expression of *ImpL2* results in the induction of *4E-BP*, a marker of insulin repression, causes non-autonomous growth inhibition and triggers phenotypes

associated with reduced insulin signalling (Honegger et al., 2008). Over-expression of *ImpL2* in the *Drosophila* eye results in reduced eye size, a reduction in the size of the whole fly and a developmental delay (Alic and Partridge, 2008), whereas loss of *ImpL2* function results in increased body size (Honegger et al., 2008). Accumulations of trehalose and organ-wasting processes are dependent on *ImpL2*, which is specifically upregulated in the proliferating midgut (Kwon et al., 2015). In *Drosophila, ImpL2* is a mediator necessary for wasting (Kwon et al., 2015). Tumour specific inhibition of *ImpL2* is sufficient to significantly ameliorate wasting phenotypes (Figueroa-Clarevega and Bilder, 2015; Kwon et al., 2015). Furthermore, a reduction in insulin signalling is responsible for mycobacterial infection induced wasting, however it is unknown if *ImpL2* is the relevant mediator in this case (Dionne et al., 2006; Figueroa-Clarevega and Bilder, 2015). In *fs(1)h* knockdown flies, *ImpL2* transcript levels were increased in whole fly samples, which may suggest *ImpL2* levels were being increased due to the increased *DILP* transcript levels and in turn may be causing wasting and playing a role in their reduced survival. Conversely, in dissected fat body there was a significant reduction in *ImpL2* transcript level following *fs(1)h* knockdown, the reason for this remains unknown. However, there may be a signal down-regulating *ImpL2* in the fat body to prevent lipid metabolism. There seems to be a range of nutrient-sensing mechanisms and pathways in both larval and adult *Drosophila* (Nässel and Vanden Broeck, 2016). *fs(1)h* may be detrimental in regulating some of these endocrine signals from the fat body and regulating energy availability.

7.7. An interaction between fs(1)h, FOXO and AKT?

The transcription factor *foxo* is a crucial mediator of insulin signalling and in response to cellular stresses, such as nutrient deprivation; FOXO is activated and inhibits growth through the activation of target genes including *4E-BP*. It has been shown that *fs(1)h* may regulate AKT activity and the level of FOXO. Reducing the level of *foxo* in *fs(1)h* fat body knockdown flies rescued many of the phenotypes observed. This may also suggest that fs(1)h could engage in a pathophysiological feedback loop, in which disturbed endocrine factors result in low insulin activity, and drive high FOXO activity. An interaction between AKT, foxo and fs(1)h is yet to be identified, however, the data obtained suggest there may be a feedback mechanism between *fs(1)h, foxo* and AKT in the insulin pathway (Figure 7-3). The possibility of an interaction between *fs(1)h* and *foxo* was strengthened further when *fs(1)h* transcript levels were increased in *foxo* homozygous null flies compared to wild-type flies.

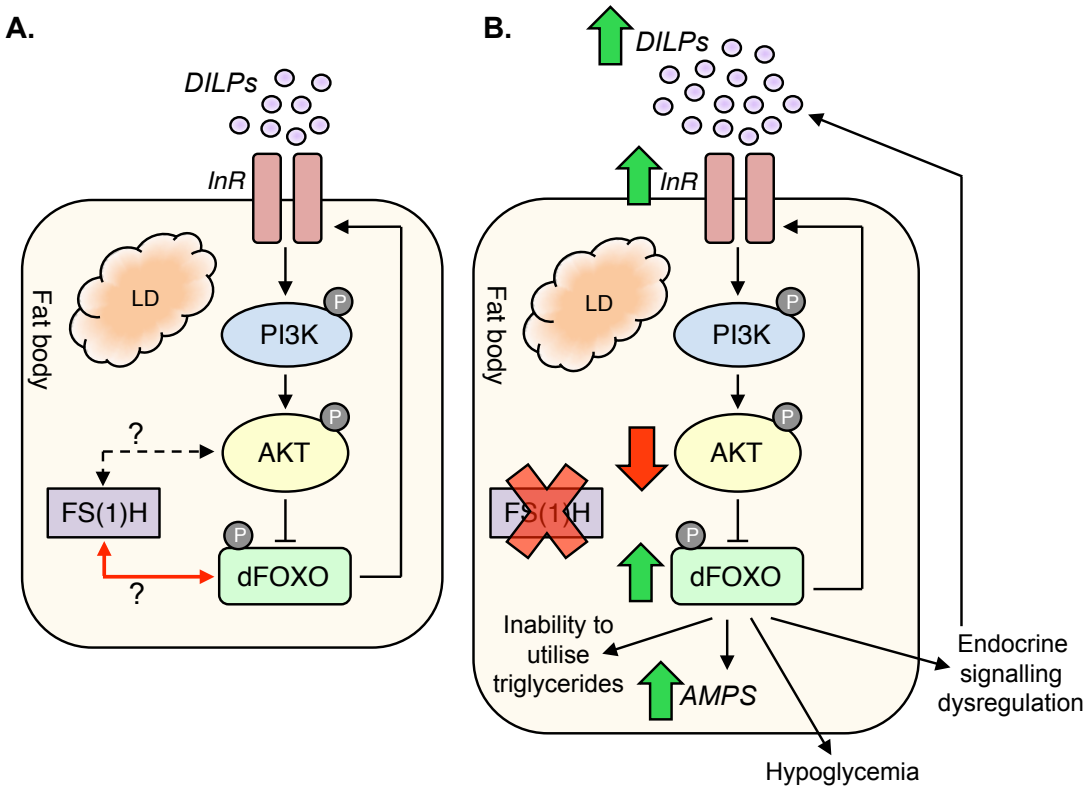


Figure 7-3: A potential interaction between fs(1)h, FOXO and AKT?
A proposed model for the role of fs(1)h in the fat body. **A.** Under physiological conditions, when *fs(1)h* is present in the fat body, the insulin-signalling pathway can function as normal. Dotted line; potential feedback mechanism and red solid line: a feedback mechanism or a potential physical or transcriptional interaction. **B.** When *fs(1)h* is lost in the fat body, the insulin signalling pathway becomes dysregulated, leading to the phenotypes observed in this thesis.

7.8. Ubiquitination dysregulation

Ubiquitin is a highly conserved, small modifier molecule that specifically labels proteins, in both physiological and pathological cellular processes (Hoeller and Dikic, 2009). The process of ubiquitination is started by ubiquitin-activating enzymes (E1), which activate and transfer ubiquitin to ubiquitin-conjugating enzymes (E2), and finally the conjugation of target proteins with ubiquitin is mediated by ubiquitin ligases (E3) (Lee et al., 2008a). Initially, ubiquitination was described as the process to label proteins for degradation by the proteasome (Hershko and Ciechanover, 1998). However, there has been an increase in functions given to different types of ubiquitin modification, such as signal transduction, assembly of protein signalling complexes, the activation or inactivation of enzymes and the regulation of protein trafficking (Bennett and Harper, 2008; Haglund and Dikic, 2005; Mukhopadhyay and Riezman, 2007). The Ubiquitin pathway has been implemented in various processes including development, the immune response and programmed cell death (Hershko and Ciechanover, 1998). Abnormalities of the ubiquitin pathway can cause pathologies,

including cancer and neurodegenerative disorders (Ciechanover and Schwartz, 2004). Following the loss of *fs(1)h* in the fat body, alterations in the abundance of proteins were observed along with a dysregulation in the ubiquitination of the whole fly proteins present.

Many proteins that are regulated by ubiquitination play roles in cellular processes including gene transcription, cell-cycle progression and apoptosis, which can all become processes involved in cancer progression and tumourigenesis (Chen and Dou, 2010; Tu et al., 2012). Additionally, ubiquitin-mediated signalling is important for activating and restricting the activity of *NF-κB* during the innate immune response, and dysregulation can lead to chronic inflammation and cancer (Chen and Chen, 2013). Following infection, pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs) trigger the activation of NF-κB by a number of signal transduction pathways (Jiang and Chen, 2011; Tigno-Aranjuez and Abbott, 2012). After the activation of NF-κB, PRRs coordinate with ubiquitination machinery to regulate *NF-κB* activation, deubiquitinating enzymes also allow for tight control of *NF-κB* (Chen and Chen, 2013). Pathogens can also counteract host defence by taking over the ubiquitin system to repress NF-κB signalling (Chen and Chen, 2013). *fs(1)h* knockdown in the fat body resulted in an increase in the abundance but not the activity of the NF-κB family member, Relish. This increase in abundance may result from a degradation defect, and in turn an accumulation of ubiquitinated protein. Therefore, *fs(1)h* may also effect processes involved in protein degradation, including autophagy or ubiquitination itself. In *Drosophila*, there is only one E1, known as *Uba1*, the E2 ubiquitin-conjugating enzyme is known as *ubcD1* and E3 is known as *DIAP1*. Trabid (*Trbd*) was originally identified as a positive regulator of the Wnt pathway in both *Drosophila* and mammals (Tran et al 2008). It has more recently been identified as a negative regulator of the *Drosophila* NF-κB pathway and interacts with Tak1, which activates the *Imd* pathway and apoptosis, to reduce immune signalling output and ubiquitination (Fernando et al., 2014). The loss of *Trabid*, much like the loss of *fs(1)h* in the fat body, results in constitutive immune activation and a dramatic reduction in lifespan in the absence of infection (Fernando et al., 2014). In the absence of infection, *Trbd* mutants also show a systemic increase in the expression of AMPs leading to a chronic response (Fernando et al., 2014). Furthermore, in mice E3 ubiquitin ligase is responsible for FOXO1 polyubiquitination and proteasome degradation in response to insulin (Huang et al., 2005). As *fs(1)h* knockdown may cause some ubiquitination dysregulation, this may also impact on increasing FOXO levels as it could not be ubiquitinated and degraded efficiently.

7.9. Human relevance

Bromodomain-containing proteins (BCPs) recognise and bind acetylated lysine residues in histone tails to alter gene expression. In recent years, the bromodomain and extraterminal domain (BET) family of BCPs have become of great interest; using small molecule pharmaceuticals to inhibit members the BET family have been extremely beneficial in the treatment of inflammation, autoimmune disease and cancer (Smith and Zhou, 2016). However, the broader *in vivo* roles of BET proteins in immunity and metabolic regulation are not entirely clear in humans or *Drosophila.* Our data from the fly suggest that BET inhibition *in vivo* may have significant metabolic consequences via AKT-FOXO signalling. Using the human monocytic cell line, THP-1, and treating with the small molecule inhibitor, I-BET151, also showed a reduction in activated AKT and an increase in FOXO levels suggesting there may be a conserved BET function in insulin signalling.

In humans, the ability for BRD2, 3 and 4 to acetylated lysine residues play a wide variety of roles in regulating chromatin function and transcription, along with their roles in cell development and cell-cycle control (Padmanabhan et al., 2016). Interestingly, when each BET protein was knocked down individually using siRNA in THP-1 cells there was no effect on AKT activation or FOXO levels, suggesting that there may be some compensation mechanism between each of the BCPs (Stonestrom et al., 2015), however this would need further investigation. This may also suggest why when *fs(1)h* is knocked down in the fat body there is such a strong phenotype as there isn't another BET protein to take over the role.

7.10. Summary

Loss of *fs(1)h*, the sole BET protein, in the *Drosophila* fat body exhibited a range of immune and metabolic phenotypes that have been shown, in most cases, to be due to the detrimental effects on AKT signalling and FOXO. Removing a single copy of *foxo* could rescue much of the uninfected metabolic phenotype, which seemed to be initiated by *foxo*-dependent dysregulation. However, during bacterial infection it could be shown that not all the *fs(1)h* phenotypes can be elucidated by FOXO hyperactivation, indicating there was also a *foxo*-independent component to the function of *fs(1)h*. We also suggest the phenotypes observed following *fs(1)h* knockdown in the fat body are a complex combination of developmental and physiological effects. The dysregulation of insulin signalling identified in *Drosophila* following *fs(1)h* knockdown could also start to be shown in the human THP-1 cell line following treatment with the BET inhibitor, I-BET151.

7.11. Future work

The role of *fs(1)h* in immunity or metabolism have not been previously reported. In order to further investigate the roles, mechanism and function of *fs(1)h* in the *Drosophila* fat body there are a number of experiments that could be carried out.

Firstly, to determine the transcriptional and epigenetic roles of *fs(1)h* in the *Drosophila* fat body experiments such as chromatin immunoprecipitation (ChIP) to determine where the fs(1)h protein is binding to DNA could be carried out. ChIP is a powerful tool for analysing histone modification and proteins that bind either directly or indirectly with DNA (Milne et al., 2009). fs(1)h is a BET protein, which recognises and binds acetylated lysine residues in histones tails, it consists of two bromodomains and an extraterminal (ET) domain involved in protein-protein interactions. Due to the known function of BET proteins and its close interaction with DNA, ChIP may provide more information as to where the bromodomains of *fs(1)h* are recognising acetylated lysine residues and binding to DNA. Furthermore, RNA sequencing (RNA-seq) is an approach to transcriptome profiling, widely used for analysing gene expression and measuring the quantities of RNA (Wang et al., 2009). RNA-seq may provide more information regarding other pathways that may be involved leading to the *fs(1)h* phenotype that has been observed. Investigating the idea of a feedback loop between *fs(1)h* and *foxo* would be extremely interesting, many of the phenotypes observed were dependent on AKT-FOXO signalling and suggest *fs(1)h* may also be playing a part in regulating insulin signalling and nutrient sensing. Additionally, *foxo* homozygous null flies had a significant upregulation of *fs(1)h* transcript level.

Using co-immunoprecipitation (Co-IP) to pulldown fs(1)h and analysing the protein-protein interactions may provide further information as to the role of *fs(1)h* in the fat body of *Drosophila* and identify potential interaction partners. It would be particularly interesting to look for a physical interaction between fs(1)h and FOXO, as this is currently unknown, however the finding in this study strongly suggest a possible interaction between them. Furthermore, cell culture and fluorescence imaging could be used to try and visualise a protein-protein interaction between fluorescently labelled fs(1)h and FOXO. Literature also suggests fs(1)h may interact with phosphatidylinositol 5-phosphate 4-kinase (PIP4K) (Rhee et al., 2014), which regulates TOR signalling and growth during *Drosophila* development (Gupta et al., 2013), so investigating that interaction further may also provide more information about *fs(1)h*. Looking into the role of TOR signalling following knockdown of *fs(1)h* in the fat body may provide further insight into its role. Our data showed reduced levels of S6 Kinase, which in mammalian cells is deactivated when amino acid availability is low (Hara et al., 1998).

Interestingly, much of our data has focused on the metabolic role of *fs(1)h* under physiological conditions and that removing one copy of *foxo* is adequate to rescue most the phenotypes observed. However, our data also showed that following infection, survival and AMP expression levels could not be rescued by *foxo* heterozygosity. Further investigation into the role of *fs(1)h* following infection with a range of pathogens may provide a further insight into its role in immunity.

Previous studies have shown that *fs(1)h* plays an important role in *Drosophila* development and body planning, and mutations in *fs(1)h* can cause segmental abnormalities. As the known role of *fs(1)h* is predominantly developmental, it would be interesting to use temperature sensitive Gal80 ($Gal80^{ts}$) in combination with the *Gal4*/UAS system to knockdown *fs(1)h* specifically in the adult fat body to decipher between developmental and non-developmental metabolic and immune phenotypes that have been observed. Although no reproducible survival phenotype was observed following the knockdown of *fs(1)h* in the hemocytes, investigating the role of *fs(1)h* in other tissues, such as the brain, gut or muscle, may provide informative and interesting results into the functions of *fs(1)h* in *Drosophila.* To further explore the role of *fs(1)h* in the immune response of the fly it may be interesting to carry out a double knockdown of *fs(1)h* in both hemocytes and the fat body. It may also be of interest to knockdown *fs(1)h* ubiquitously or use *fs(1)h* mutants to explore the total loss of *fs(1)h* in *Drosophila* compared to tissue specific knockdowns.

Drosophila lacking *fs(1)h* in the fat body appear to show dysfunction in ubiquitination, which may cause some of the phenotypes observed. Further investigation into how *fs(1)h* is affecting ubiquitination would be of interest. This could be done by looking at transcript levels of the ligase and proteases involved in ubiquitination in *Drosophila* and trying to rescue any of the observed phenotypes by over-expressing *Trabid*, which plays important roles in ubiquitination and reducing immune signalling output.

Finally, *Drosophila* are a robust model in therapeutic drug discovery and chemical screening, feeding or injecting BET inhibitors into the flies to try and reproduce the *fs(1)h* phenotypes observed following RNAi knockdown in the fat body would be interesting. Carrying out more experiments using BET inhibitors, to treat THP-1 cells and other human cell lines may provide insightful information about the conservation of BET proteins between in *Drosophila* and humans, along with increasing the knowledge for treatment of human disease.

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